ISOLATION AND CHARACTERIZATION OF A VARIANT HUMAN H2B HISTONE GENE EXPRESSING ALTERNATIVE mRNAs REGULATED DIFFERENTIALLY DURING THE CELL CYCLE AND DIFFERENTIATION

By

DAVID GEORGE COLLART

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
1991

Dedicated to Mary,

the mother of God.

ACKNOWLEDGEMENTS

I would like to thank Drs. Gary and Janet Stein for their support and guidance during my graduate studies. Their dedication as scientists has provided us with a laboratory which has always been well equipped and supplied, allowing us to carry out the experiments necessary for the completion of our individual projects. In addition, I would like to thank Gary and Janet for all their support on a personal level during all the trials which life has offered throughout my journey from a single, and sometimes foolhardy, new graduate student to a married, and hopefully more mature, scientist. I would also like to thank Drs. Harry Nick, Philip Laipis, Harry Ostrer, Carl Feldherr, Lee Weber and Eileen Hickey for their helpful advice as members of my graduate committee. I am also grateful for the support and help of Dr. Richard Boyce. In addition, I thank Anna Ramsey-Ewing, Dr. Paul Romain, Suzie Pilapil, Dr. Jane Lian, Shirwin Pockwinse, Dr. Rita Bortell, Dr. Carlo Croce, Dr. Kay Huebner and Dr. Linda A. Cannizzaro for their collaborative efforts on various aspects of this research. I would like to acknowledge Christine Dunshee for her photography.

I would like to thank all of the members of the Steins' lab for their companionship over the years. I thank Linda Green, Farhad Marashi and Mark Plumb for their friendship and helpful discussions during the early years of my graduate studies. I thank André van Wijnen for his friendship and helpful suggestions on writing manuscripts and Anna Ramsey-Ewing for her friendship and scientific expertise in tissue culture and transcriptions. I would also like to thank Tim Morris for his friendship and discussions on everything in the heavens and on earth, and Gerry Zambetti for his friendship and for being the godfather of little Timothy Gerard. In addition, I thank all my roommates during my pre-marriage days in particular Charlie for his Scottish outlook on life and money, and Andy for all of his friendship and support during my years as a graduate student.

Finally, I would like to thank my family. My parents and brothers and sisters have always supported me and have taught me so much about caring. I thank my wife Kathy, whose prayers and constant love have always been a source of joy and strength for me. I thank our son Timothy, whose smiles, giggles and squiggles have taught me what happiness is and for his suggested addition to this dissertation, "aah ahh ook." Lastly, I give thanks to God for his constant love, forgiveness and blessings.

TABLE OF CONTENTS

LIST OF FIGURES

KEY TO ABBREVIATIONS

APS:	Ammonium Persulfate
ATP:	Adenosine-5'-triphosphate
bp:	Base pair
BSA:	Bovine serum albumin
CIP:	Calf intestinal alkaline phosphatase
°C:	Degree centigrade
DMEM:	Dulbecco's minimal essential medium
dATP:	2'-Deoxyadenosine 5'-triphosphate
dCTP:	2'-Deoxycytidine 5'-triphosphate
dGTP:	2'-Deoxyguanosine 5'-triphosphate
DNase I:	Deoxyribonuclease I
DNA:	Deoxyribonucleic acid
DEPC:	Diethyl pyrocarbonate
dpm:	Disintegrations per minute
DTT:	Dithiothreitol
ddH_2O:	Double-distilled water
EDTA:	Ethylenediaminetetraacetic acid
g:	Gram
g:	Gravitational force
GAPDH:	Glyceraldehyde-3-phosphate-dehydrogenase
HBSS:	Hank's balanced salt solution
IAA:	Isoamyl alcohol

IPTG:	Isopropyl-β-D-thiogalactopyranoside
l:	Liter
μg:	Microgram
μm:	Micrometer
mg:	Milligram
ml:	Milliliter
mm:	Millimeter
mM:	Millimolar
M:	Molar
MOPS:	Morpholinepropane-sulfonic acid
ng:	Nanogram
nt:	Nucleotide
OD:	Optical density
PBS:	Phosphate buffered saline
pfu:	Plaque forming unit
Poly (A):	Polyadenosine
PEG:	Polyethylene glycol
PVP:	Polyvinyl-pyrrolidone
PVS:	Polyvinylsulfonic acid
RF:	Replicative form
RNase A:	Ribonuclease A
RNA:	Ribonucleic acid
rpm:	Revolutions per minute
SDS:	Sodium dodecyl sulfate
TCA:	Trichloro-acetic acid
TEMED:	N,N,N',N'-tetramethylethylenediamine

tH2B:	Testis specific H2B
TPA:	12-O-tetradecanoyl phorbol-13-acetate
TTP:	Thymidine 5'-triphosphate
U:	Unit of enzyme
X-gal:	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of
the Requirements for the Degree of Doctor of Philosophy

ISOLATION AND CHARACTERIZATION OF A VARIANT HUMAN H2B HISTONE GENE EXPRESSING ALTERNATIVE mRNAs REGULATED DIFFERENTIALLY DURING THE CELL CYCLE AND DIFFERENTIATION

By

David George Collart

May 1991

Chairman: Gary Stein
Major Department: Biochemistry and Molecular Biology

We have isolated and characterized a cDNA (λHHC289) corresponding to a variant human histone H2B gene. The H2B protein coding region of λHHC289 is flanked at the 3' end by a 1798 nt non-translated trailer that contains a region of hyphenated dyad symmetry and a poly(A) tail. Nuclear run-on transcription analysis revealed a two fold increase in transcription of the HHC289 gene during S phase. Northern blot analysis indicated that the levels of the 2300 nt HHC289 mRNA species were constant during the HeLa cell cycle. Northern blot analysis also revealed that the levels of the 2300 nt HHC289 H2B species increased 10-fold during HL60 cell differentiation whereas the levels of replication-

dependent H2B mRNAs decreased to less than 1% of those in proliferating cells.

Using a probe from the λHHC289 cDNA that detects only a single copy region of the human genome we isolated a variant human H2B histone gene (GL105) which expresses alternative mRNAs regulated differentially during the HeLa cell cycle. This H2B gene encodes both a 500 nt replication-dependent mRNA and the 2300 nt HHC289 constitutively expressed mRNA. The 3' end of the cell cycle regulated mRNA terminates immediately following the region of hyphenated dyad symmetry typical of most histone mRNAs, whereas the constitutively expressed HHC289 mRNA has a 1798 nt non-translated trailer that contains the same region of hyphenated dyad symmetry but is polyadenylated. The cap site for both H2B-GL105 mRNAs is the same and is located 42 nt upstream of the protein coding region. The H2B-GL105 histone gene was localized to chromosome region 1q21-1q23 by chromosomal in situ hybridization of a H2B-GL105 specific probe and by analysis of rodent-human somatic cell hybrids. The H2B-GL105 gene is paired with a functional H2A histone gene and this gene pair is separated by a bidirectionally transcribed intergenic promoter region containing consensus TATA and CCAAT boxes and an OTF-1 element. These results demonstrate that replication-dependent and constitutively expressed histone mRNAs can be encoded by the same gene and indicate that alternative 3' end processing is a major level

of regulation by which cells can modulate the synthesis of variant histone proteins during the cell cycle and at the onset of differentiation.

CHAPTER 1

INTRODUCTION

General Background

All cells of higher animals and plants carry genetic information in chromosomes in the form of long linear molecules called DNA. Within these DNA molecules are regions containing units of genetic information, called genes, whose expression results in the production of proteins or RNA molecules necessary for cellular function. In the human being more than 1 meter of DNA is contained within the cell nucleus. DNA must be packaged efficiently within the cell in such a manner that it can be replicated when the cell divides, and transcribed at most stages throughout the cell cycle. This packaging process is carried out primarily by a group of proteins, approximately equal in mass to the DNA itself, called histone proteins. When DNA is associated with histone proteins it is called chromatin.

When a cell divides, the DNA within a cell is replicated and the cell must therefore also double the amount of its histone protein. Because the cell requires a large quantity of histone protein to package its DNA, it

must carefully regulate the production of these proteins according to the cellular needs. The cell must allow for production of large amounts of histone protein during periods of rapid growth and division but when DNA replication ceases, such as outside of S phase or at the onset of differentiation, the cell must downregulate histone production to a low level but sufficient to provide for replacement of histone proteins and for reorganization of chromatin. Therefore, the study of histone gene expression provides an excellent opportunity to understand the changes in gene expression during the transition from a proliferative to a nondividing state.

Histone Proteins

Histones are a complex family of highly conserved basic proteins responsible for packaging chromosomal DNA into nucleosomes (85,86). There are five major classes of histone proteins (H1, H2A, H2B, H3, and H4) (85,86). Histone proteins exhibit two levels of diversity: (i) evolutionary diversity between species and (ii) subtype diversity in a class (H1, H2A, H2B, H3, or H4) within a species (164). The subtypes within a species are often referred to as variants (187,188,195). Replication-dependent histone variants represent the majority of the histone proteins synthesized in proliferating cells, and it is well established that their synthesis is functionally and temporally coupled to

DNA replication (1,133,164). Replacement variants are expressed in nonproliferating cells and are often expressed in proliferating cells, although normally at a lower level than their replication-dependent counterparts (188,195). The third type of variant within a species is the tissue-specific variant (17,40,83,84,172). Tissue-specific variants are generally not synthesized in a replication-dependent manner (40,83,84).

Histone H2B proteins have been studied in a variety of species--including chicken, mouse, rat and human--with respect to their subtype diversity (17,88 and reviewed in 164,195). Although the replication-dependent and tissue-specific H2B proteins are easily detected in most species, the replacement histone H2B proteins are not always observed in humans, possibly due to the lack of sensitivity of the assay (164). In mouse the H2B.2 variant is the replication dependent subtype and is abundant in rapidly dividing cells (195). The H2B.1 variant is the constitutively expressed subtype and is present in small amounts in rapidly growing tissues but increases sharply as proliferation slows, especially after the postnatal growth phase (195). The H2B subtype specific amino acids of the H2B.1 mouse protein (Ser^{14}, Ala^{21}, Asp^{25}, Lys^{27}, Gly^{60}, Asp^{68}, Gly^{75}, Glu^{76}) differ from those of the H2B.2 protein only at Ser^{75} (195), however, the functional significance of this change is unclear. In general, replacement histone variants are less

hydrophobic than replication-dependent or tissue specific variants.

Structure and Organization of Human Histone Genes

Human histone genes are a family of moderately reiterated sequences (75,91,154,164) and are arranged in clusters; they show no evidence of a simple tandem repeat organization (75,154,164,194) as do the histone genes of sea urchin species (65,164,186) and Drosophila (102,164). In humans, these clusters are localized on chromosomes 1, 6, and 12 (62,164,171) and are associated with a series of repetitive DNA sequences (36). Cell cycle dependent histone genes do not contain introns (91,164), and they encode mRNAs which are not polyadenylated and have short 5' leader and 3' trailer sequences (77,164). Although replication-dependent histone mRNAs are structurally simple, they do have a characteristic 3' stem-loop motif (164). In contrast, constitutively expressed histone genes are structurally more complex. They may contain introns (20,43,182,183,185), and they encode mRNAs which are polyadenylated and frequently contain long 5' leader or 3' trailer sequences (16,20,28,45, 46,71-73,78,96,100,110,116,143,144,175,182,183).

Coupling of DNA Synthesis and Histone Gene Regulation

Replication-dependent histone genes are coordinately expressed during the cell cycle and their expression is

coupled with DNA synthesis (3,74,126,130,131). The abundance of their mRNAs is regulated at both the transcriptional and post-transcriptional levels (3,74,130,131,176 and reviewed in 113,150,151,163,164). In comparison, the mRNAs for replacement histones (10,19,25,45,71,89,152,155) as well as tissue-specific histone mRNAs (27,34,64,83,84,93) are not always regulated in a replication-dependent manner.

Transcriptional and Post-Transcriptional Regulation of Human Histone Genes

Transcriptional regulation of human histone genes involves a 2 to 5 fold enhancement in the rate of transcription within the first 2 hours of DNA synthesis followed by a return to basal level by mid to late S phase (4,74,113,131,151,163). However, steady state levels of histone mRNA are elevated 15-to 20-fold during S phase of the cell cycle and parallel the rates of DNA synthesis (3,74,130,131,176). Although the rate of histone gene transcription peaks within the first 2 hours of DNA synthesis, histone mRNA levels do not reach a maximum until 4-5 hours into S phase. Taken together these observations suggest post-transcriptional mechanisms play a role in the regulation of histone mRNA levels.

Post-transcriptional regulation of histone genes involves a rapid and selective destabilization of histone mRNAs toward the end of S phase or in response to inhibition

of DNA synthesis (3,76,117,127,128,130,142). However, there is little or no effect on histone gene transcription by inhibition of DNA synthesis (4,130,155). This destabilization of histone mRNA upon inhibition of DNA synthesis is dependent upon protein synthesis (3,22,76). In contrast, the mRNA levels of replication-independent histone genes are not regulated in the same cell cycle dependent manner as their replication-dependent counterparts and remain relatively constant upon inhibition of DNA synthesis (10,19,25,35,45,71,89,152).

The 3' end of cell cycle dependent mRNAs contains a region of hyphenated dyad symmetry (77) which is essential, although not sufficient, for coupling histone mRNA stability with DNA replication (2,101,106,125,159). Graves et al. (61) observed that moving the 3' stem-loop motif affects coupling of histone mRNA stability with DNA replication. Destabilization of histone mRNA initiates at the 3' terminus (140,142), and is continued rapidly in a 3' to 5' direction by a ribosome complex-associated endonuclease (141). In addition, subcellular localization may play a role in histone mRNA stability (192,193).

The 3' end of most eukaryotic mRNAs is formed by an endonucleolytic cleavage reaction followed by polyadenylation of the 3' terminus (12,53,122,129). This cleavage reaction requires two sequences, a highly conserved AAUAAA sequence and a downstream GU rich sequence, which

flank the cleavage site (12,53,122,129,179). Most histone mRNAs are not polyadenylated and their mRNA 3' end formation requires two elements: the stem-loop motif and a purine rich sequence 3' to the stem-loop motif (9,12,159,178). An endonucleolytic cleavage reaction occurs 3' to the stem-loop motif (12,55,95,118,167) and is dependent upon the interaction of the U7 snRNA with the purine rich sequence (12,39,118-120,156,167).

H2B Histone Gene Expression

To date, H2B histone gene organization and expression have been studied most thoroughly in the chicken; five replication-dependent, one partially replication-dependent, one uncharacterized and one testis-specific chicken H2B genes have been described (27,59,60,83,84). Human H2B histone gene expression has not been reported in such detail. Although several human histone H2B replication-dependent genes and one pseudogene have been described (74,112,131,134,154,164,194), no human replacement or tissue-specific H2B genes have been reported.

Overview of Project

The overall aim of this work was to isolate and characterize a variant human histone gene and study its regulation in response to changes in the proliferative state of the cell. When we began these studies, information in

the literature on nonhuman variant histone genes indicated the genes for human variant histone proteins would be structurally more complex than their replication-dependent counterparts (20,43,71,185). This indication was supported by the observations of Borun et al. (16) that a fraction of human histone mRNAs contained short tracts of poly(A). In addition, because constitutively expressed histone proteins are synthesized throughout the cell cycle (164,188,195), their mRNAs must be stable in the absence of DNA synthesis. Presumably, structural features of constitutively expressed histone mRNAs could render them resistant to the mechanisms which rapidly and selectively destabilize replication-dependent histone mRNAs toward the end of S phase or in response to inhibition of DNA synthesis (3,76,117,142). Alternatively, the absence of structural elements endogenous to replication-dependent histone mRNAs could have the same result. Therefore, we have focused our attention on the cloning of a variant human H2B histone gene, and characterization of its structural features and their effect on expression in response to changes in the growth state of the cell.

CHAPTER 2

MATERIALS AND METHODS

Materials

Materials and Biochemical Reagents

The majority of chemicals, reagents and solvents were purchased from either Fisher Scientific, Springfield, NJ or Sigma Chemical Co., St. Louis, MO., unless otherwise indicated. Formamide and X-gal were purchased from Bethesda Research Laboratories (BRL), Gaithersburg, MD. Bio-Gel A-1.5m, A-5m, and A-15m (100-200 mesh) agarose beads for gel filtration, Dowex 50W-X8 cation exchange resin (200-400 mesh) hydrogen form, ultrapure electrophoresis grade agarose and Zeta-probe nylon membranes were purchased from Bio Rad, Richmond, CA. Acrylamide (crystallized), APS, ultrapure bis-acrylamide (N,N'-methylene-bis-acrylamide), DTT (Cleland's reagent), glycogen (molecular biology grade), and IPTG were purchased from Boehringer Mannheim Biochemicals (BMB), Indianapolis, IN. Cronex x-ray film was manufactured by Dupont, Wilmington, DE. Dimethyl sulfate, dichlorodimethyl silane, hydrazine, PEG 8000, PVS, XAR-5 x-ray film and X-Omat duplicating film were manufactured by Eastman Kodak Co., Rochester, NY. SPECTRA/POR

molecularporous membrane tubing, cellusolve (ethylene glycol monoethyl ether), dimethyl sulfoxide, ethidium bromide, 2-piperidine, TCA and toluene were purchased from Fisher Scientific, Springfield, NJ. Cesium chloride (special biochemical grade 99.9%) was purchased from Gallard-Schlesinger Industries Inc., Carle Place, NY. OPTI-FLUOR scintillation cocktail was purchased from Packard Instrument Co., Inc., Downers Grove, IL. Ficoll 400 was purchased from Pharmacia Inc., Piscataway, NJ. Type 55 and type 57 instant sheet film were purchased from Polaroid, Cambridge, MA. Concentrated liquid scintillator (Liquifluor) was purchased from Research Products International, Elk Grove Village, IL. Elutip-d mini-columns, 82 mm and 132 mm nitrocellulose circles (pore: 0.45 μm) and nitrocellulose sheets were purchased from Schleicher and Schuell, Inc., Keene, NH. BSA (fraction V), DEPC, N,N-dimethyl formamide, PVP and TEMED were purchased from Sigma Chemical Co..

Antibiotics, Growth Factors, Inhibitors and Media

Antibiotics and media were prepared as described in appendix A. Fetal bovine, calf bovine and horse sera were obtained from Gibco Laboratories, Grand Island, NY or Flow Laboratories, McLean, VA. Maltose was purchased from Becton Dickinson and Co., Cockeysville, MD. Bacto-agar, bacto-tryptone, bacto-yeast extract, and vitamin-free casamino acids were purchased from Difco Laboratories, Detroit, MI.

Dextrose was purchased from Fisher Scientific, Springfield, NJ. Geneticin (G418 sulfate) was purchased from Gibco Laboratories. Chloromycetin sodium succinate (chloramphenicol sodium succinate) was purchased from Parke-Davis, Morris Planes, NJ. TGF-β1 was purchased from R&D Systems, Inc., Minneapolis, MN. Ampicillin (D[-]-α aminobenzylpenicillin) sodium salt, dexamethasone, hydroxyurea, insulin (from bovine pancreas), 3-isobutyl-1-methyl-xanthine, NZ amine (casein enzymatic hydrolysate), penicillin-G (Benzyl penicillin) potassium salt, streptomycin sulfate (streptomycin sesquisulfate), tetracycline, TPA and trypsin (from porcine pancreas) prepared in 1X HBSS without calcium or magnesium were purchased from Sigma Chemical Co.

Enzymes and Kits

Restriction endonucleases were purchased primarily from BRL, BMB, and New England Bio Labs (NEB), Beverly, MA. Enzyme reactions were carried out using the buffer and incubation conditions described by the manufacturers unless otherwise indicated. *E. coli* DNA polymerase I large fragment (Klenow enzyme) was purchased from BRL. Proteinase K, CIP, nuclease S1 and T4 DNA ligase were purchased from BMB. Trypsin (0.25% in buffered saline) was purchased from Hazleton Biologics, Inc., Lenexa, KS. The EXOMETH DNA sequencing kit with reverse transcriptase and

the PRIME-IT random primer kit were purchased from Stratagene, La Jolla, CA. DNase I (DN-EP), DNase I (DN-25) and RNase A (Type III-A) were obtained from Sigma Chemical Co.. The Sequenase 2.0 DNA sequencing kit and T4 polynucleotide kinase (cloned) were from United Stated Biochemical Corp. (USB), Cleveland, OH. Lysozyme was purchased from Worthington Biochemicals, Freehold, NJ.

Nucleic Acids and Nucleotides

[methyl-^{3}H]Thymidine (20.0 Ci/mmole), [α-^{32}P]dATP (~3,000 Ci/mmole), [α-^{32}P]dCTP (~3,000 Ci/mmole), [γ-^{32}P]ATP (~3,000 Ci/mmole), [α-^{32}P]UTP (~3,000 Ci/mmole) and [α-^{35}S]dATP (>1,000 Ci/mmole) radionucleotides were purchased from Amersham Corp., Arlington Heights, IL, or NEN Research Products, Wilmington, DE. Oligonucleotides were synthesized in the recombinant DNA core facility of the Department of Cell Biology at the University of Massachusetts Medical Center using an Applied Biosystems (AB) 380A DNA synthesizer purchased from AB, Foster City, California. ATP (from equine muscle), dATP (sodium salt), dCTP (sodium salt), dGTP (sodium salt), TTP (sodium salt), oligo dT-cellulose and salmon testes DNA (type III) were obtained from Sigma Chemical Co. RNA molecular weight markers were purchased from BMB and from BRL. M13mp18 RF DNA and M13mp19 RF DNA were obtained from BMB.

Propagation and Maintenance of Bacterial Strains

Growth of Bacterial Strains

Escherichia coli (*E. coli*) bacteria (see appendix C) were grown at 37°C in YTN medium with vigorous shaking or on plates containing YTN bottom agar (see appendix A) unless otherwise indicated. If necessary, ampicillin was added to the media to a final concentration of 50 μg/ml or tetracycline to 15 μg/ml immediately prior to inoculation of liquid cultures or pouring of YTN agar plates. Liquid cultures of bacteria were usually grown such that the volume of the airspace above the culture was at least 3 times that of the medium, unless otherwise indicated.

Storage of Bacterial Strains

Bacterial colonies were stored for short periods of time on the surface of bottom agar plates at 4°C. These plates were wrapped in parafilm to prevent moisture loss and stored inverted.

For long-term preservation of bacterial strains bacteria were stored in medium containing 50% glycerol at -70°C. To prepare bacterial stocks, 5 ml of YTN was inoculated with a single colony of bacteria and grown anaerobically for 18 hours. A 0.5 ml aliquot of the anaerobic culture was then placed into a vial containing 0.5 ml of sterile glycerol, the contents were mixed and the vial stored at -70°C.

Propagation and Maintenance of λ Bacteriophage

Growth and Titration of λ Bacteriophage

Lambda Charon 4A (13) and λEMBL4 (54) recombinant phage were grown in the LE392 strain of *E. coli* (15,44,121) and λgt11 recombinant phage in the Y1088 strain of *E. coli* (191). Lambda bacteriophage were grown either on NZCYM plates as described by Maniatis *et al.* (108), in small scale liquid cultures as described by Leder *et al.* (98) or in large scale liquid cultures (13,109).

Preparation of plating bacteria (108). A single bacterial colony (or 1 loop of bacteria from a glycerol stock) was used to inoculate 50 ml of NZCYM medium, supplemented with 500 μl of 20% maltose (see appendix A), and grown for 10-15 hours at 37°C with vigorous shaking. Maltose induces the maltose operon, containing the gene coding for the λ receptor which is essential for efficient absorption of λ bacteriophage to bacteria. The cells were then centrifuged at 3000 rpm in an IEC rotor (2000 X g) for 5 minutes at 4°C. The supernatant was discarded and the cell pellet resuspended in 20 ml (0.4 X the volume of the original culture) sterile 10 mM $MgSO_4$. These bacteria were stored at 4°C and used for up to 3 weeks; however, normally the cells were used within 1-2 days of their preparation.

Obtaining a titer of λ bacteriophage. Titration of bacteriophage was carried out essentially as developed by Felix d'Hérelle in 1920 (165) and described by Maniatis et al. (108). Serial dilutions of bacteriophage were prepared in SM and 100 μl aliquots of each dilution to be assayed were placed into 13-mm x 100-mm culture tubes. A 100 μl aliquot of plating bacteria was placed into each tube, and the tube was vortexed gently and incubated at 37°C for 15-20 minutes to allow the bacteriophage to absorb. Upon completion of the incubation period, 4 ml aliquots of NZCYM top agar (45°C) were added to each tube, and the contents were vortexed gently and then poured onto NZCYM bottom agar plates (see appendix A for preparation of NZCYM top agar and NZCYM bottom agar plates). Upon solidification of the top agar the plates were inverted and incubated at 37°C for 12-18 hours to allow plaque formation.

Small scale liquid cultures of λ bacteriophage (98). Approximately 1/3-1/2 of a resuspended bacteriophage plaque or 3 X 10^6 bacteriophage from a stock was mixed with 100 μl of a fresh bacterial overnight culture in a sterile, 50-ml polypropylene tube and incubated for 20 minutes at 37°C. Following absorption of the bacteriophage, 4 ml of NZCYM medium was added and the tube incubated at 37°C with shaking for 6-12 hours until lysis occurred. After lysis, 40 μl of $CHCl_3$ was added and the incubation continued for 15 minutes.

Upon completion of the incubation period, the culture was centrifuged at 5800 rpm in a Beckman JA20 rotor (4000 X g) for 10 minutes at 4°C. Next, the supernatant was recovered, 12 μl of $CHCl_3$ added and the stock stored at 4°C. The titers were normally in the range of 10^8-10^{10}/ml.

Large scale liquid cultures of λ bacteriophage (13,109). A single bacterial colony (or 1 loop of bacteria from a glycerol stock) was used to inoculate 100 ml of NZCYM medium, which was grown for 10-15 hours at 37°C with vigorous shaking. The OD_{600} of the culture was measured and the cell concentration calculated, assuming that 1 OD_{600}=8 X 10^8 cells/ml (108). An aliquot containing 10^{10} cells was removed and centrifuged at 3000 rpm in an IEC rotor (2000 X g) for 5 minutes at 22°C. After centrifugation the supernatant was discarded and the cells were resuspended in 3 ml SM. Approximately 5 X 10^8 bacteriophage were added to the cell suspension and the tube was incubated at 37°C for 20 minutes with intermittent shaking. Upon completion of the incubation period, the contents of the tube were added to 500 ml of prewarmed NZCYM and incubated at 37°C with vigorous shaking for 7-12 hours until lysis occurred. After lysis, 10 ml of $CHCl_3$ was added and the incubation continued for 30 minutes. Upon completion of the incubation, the λ bacteriophage were purified as described by Yamamoto et al. (189).

Isolation and Purification of λ Bacteriophage (189)

After treatment of the large scale preparation of λ bacteriophage with $CHCl_3$, the culture was chilled to room temperature, pancreatic DNase I and RNase A were added to a final concentration of 1 μg/ml each and the culture was incubated for 30 minutes at room temperature. Upon completion of the incubation, solid NaCl was added to a final concentration of 1 M (29.2 g), dissolved by swirling and the culture incubated for 1 hour on wet ice. The culture was then poured into a 500 ml centrifuge bottle, the $CHCl_3$ was carefully left behind, and the culture centrifuged at 9500 rpm in a Beckman JA10 rotor (11,000 X g) for 10 minutes at 4°C. The supernatant was poured into a clean flask; solid PEG 8000 from Eastman Kodak (chemical source is important) was added to a final concentration of 10% w/v (50 g), dissolved by slow stirring, and incubated for at least 1 hour on wet ice (often this precipitation step was carried out at 4°C overnight). After the incubation, the precipitated bacteriophage particles were recovered by centrifugation at 9500 rpm in a Beckman JA10 rotor (11,000 X g) for 10 minutes at 4°C. The supernatant was poured off and the bottles were drained in a tilted position for 5 minutes. The bacteriophage pellet was gently resuspended in 8 ml of SM, using a Pasteur pipette and a rubber bulb to dislodge the bacteriophage particles from the wall of the centrifuge bottle. The bacteriophage suspension

was extracted with 8 ml $CHCl_3$/IAA (24:1, v/v) and centrifuged at 2500 rpm in an IEC rotor (1400 X g) for 15 minutes at 4°C. The aqueous phase was recovered and the above extraction repeated until no PEG interface remained. Upon completion of the $CHCl_3$ extractions 0.75 g of solid CsCl was added for every ml of solution and mixed gently. The bacteriophage suspension was then centrifuged at 38,000 rpm in a Beckman 50Ti or 70.1Ti rotor (135,000 X g) for 24 hours at 4°C. The band of bacteriophage particles was collected using an 18 gauge needle and stored at 4°C.

Storage of λ Bacteriophage Stocks

Bacteriophage were stored in one of three ways: (i) Pasteur pipette plugs of bacteriophage plaques were placed into 1 ml aliquots of SM (initial titers of approximately 10^6-10^7/ml) and stored at 4°C for 2-4 years, (ii) supernatants obtained from small scale liquid cultures of bacteriophage (initial titers of approximately 10^8-10^{10}/ml) were stored at 4°C for 3-5 years, and (iii) suspensions of bacteriophage in CsCl, obtained from large scale liquid cultures of bacteriophage purified over CsCl gradients (initial titers of approximately 10^{10}-10^{11}/ml), were stored at 4°C for over 5 years. Stocks of bacteriophage generally experienced a drop in titer of one order of magnitude during the first month of storage and an equivalent drop during each year of further storage.

Recombinant Phage DNAs

The λ Charon 4A recombinant bacteriophage used in this study were previously isolated (24,154,196) from a λ Charon 4A genomic DNA library. Also used in this study were λgt11 recombinant bacteriophage isolated (see chapter 3) from a λgt11 human liver cDNA library and λEMBL4 recombinant bacteriophage which were isolated (see chapter 4) from a λEMBL4 adult human lymphocyte genomic DNA library.

Isolation and Purification of Bacteriophage DNA

Large Scale λ Bacteriophage DNA Isolation (109)

DNA was isolated from purified λ bacteriophage as described by Maniatis et al. (109). The purified bacteriophage preparation was dialyzed twice at room temperature for 1 hour against a 1000 X volume of 10 mM NaCl, 50 mM Tris•Cl (pH 8.0), 10 mM $MgCl_2$, to remove cesium chloride. The bacteriophage suspension was transferred to a glass, 50 ml extraction tube and EDTA added to a final concentration of 20 mM. Proteinase K was added to a final concentration of 50 μg/ml, SDS to a final concentration of 0.5% (w/v) and the solution was mixed gently and incubated at 65°C for 1 hour. The solution was extracted with an equal volume of equilibrated phenol (see appendix B) and centrifuged at 2500 rpm in an IEC rotor (1400 X g) for 10 minutes at room temperature. Next, the solution was extracted with an equal volume of a 1:1 mixture (v/v) of

equilibrated phenol and $CHCl_3$/IAA (24:1, v/v) and centrifuged at 2500 rpm in an IEC rotor (1400 X g) for 10 minutes at room temperature. The aqueous phase was recovered and extracted once with an equal volume of $CHCl_3$/IAA (24:1,v/v). Upon completion of the extractions the aqueous phase was transferred to a dialysis sac and dialyzed sequentially for 6 hours, at 4°C, against 3 1000 X volumes of 10 mM Tris•Cl, 1 mM EDTA (pH 8.0). The purified DNA was quantitated and stored at either 4°C or -20°C.

Small Scale λ Bacteriophage DNA Isolation (149)

Small scale λ bacteriophage DNA preparations were carried out as described by Schweizer (149). A high titer lysate of λ bacteriophage, 0.5 ml, was transferred to a 1.5 ml microcentrifuge tube containing 10 μl 10% SDS (w/v). Subsequently, 1 μl DEPC was added and the tube mixed by inversion. Next, 100 μl of 1 M Tris•Cl (pH 8.0) and 25 μl 0.5 M EDTA were added, the tube mixed again by inversion and incubated for 5 minutes at 70°C. After the incubation, 80 μl 3 M sodium acetate (pH 5.2) was added and the incubation continued for 30 minutes on wet ice. The solution was then centrifuged at 12,000 X g for 15 minutes at 4°C. Following centrifugation, the supernatant was decanted to a fresh microcentrifuge tube. The tube was filled with 95% ethanol (-20°C), mixed by inversion and centrifuged at 12,000 X g for 5 minutes at room temperature.

The supernatant was discarded and the pellet washed with 1.0 ml 70% ethanol at room temperature. The pellet was dried briefly under vacuum and resuspended in 200 µl TES buffer (20 mM Tris•Cl (pH 7.5), 10 mM NaCl, 0.1 mM EDTA). The DNA was applied to a 2 ml column of Bio-Gel A-0.5m agarose beads and eluted with TES buffer. Fractions were collected and analyzed on a 1% agarose plate containing 5 µg/ml ethidium bromide. Fractions containing λ bacteriophage DNA were pooled and precipitated with 0.3 M NaCl and 2.5 volumes of 95% ethanol (-20°C), mixed by inversion and centrifuged at 12,000 X g for 5 minutes at room temperature. The supernatant was discarded and the pellet washed with 1.0 ml 70% ethanol at room temperature. The pellet was dried briefly under vacuum, resuspended in 50 µl 10 mM Tris•Cl (pH 8.0), 1 mM EDTA containing DNase-free RNase (50 µg/ml) and incubated for 30 minutes at 37°C. Following incubation the DNA was stored at -20°C.

Single-Stranded M13 Bacteriophage Template Isolation

Single-stranded M13 Bacteriophage templates for sequencing cloned inserts were prepared as described by Sanger et al. (146) and Messing (115). An exponentially growing culture of host cells, XL1-Blue (21) (see appendix C), was diluted 1:100 in 2 ml of YTN medium, infected with a purified M13 plaque and incubated for 6-8 hours at 37°C with vigorous shaking. Following the

incubation, 1.5 ml of the culture was transferred to a microcentrifuge tube and spun in an Eppendorf centrifuge at 12,000 X g for 10 minutes at 4°C. The supernatant (1.2 ml) was transferred to a microcentrifuge tube containing 300 μl of 20% PEG, 2.5 M NaCl and incubated at room temperature for 15 minutes. The remaining supernatant was placed into a clean microcentrifuge tube and stored at -20°C as a phage stock. After the incubation, the phage suspension was centrifuged at 12,000 X g for 5 minutes at room temperature and the supernatant was carefully removed and discarded. The phage pellet was resuspended in 150 μl TES buffer (20 mM Tris•Cl (pH 7.5), 10 mM NaCl, 0.1 mM EDTA) by vortexing for 2 seconds. Equilibrated phenol (50 μl) was added to the virus suspension, vortexed for two seconds, incubated at room temperature for 5 minutes, vortexed for an additional 2 seconds and centrifuged at 12,000 X g for 4 minutes at room temperature. Single-stranded template DNA was precipitated from 130 μl of aqueous phase with 5 μl 3 M sodium acetate, 1 μl glycogen (20 mg/ml) and 405 μl 95% ethanol on crushed dry ice for 15 minutes and pelleted by centrifugation at 12,000 X g for 30 minutes at 4°C. The supernatant was decanted and the pellet washed with 100 μl 70% ethanol at room temperature. The DNA pellet was dried under vacuum, resuspended in 80 μl TES buffer and stored at -20°C.

Recombinant Plasmid DNAs

DNA fragments containing H1, H2A, H2B, H3 and H4 genes from recombinant λ Charon 4A phage (24,154,196) were subcloned into pBR322 or pUC8 ((131,154,196) and unpublished data). The plasmid RGAPDH-13 (52) was generously provided by Dr. Ph. Jeanteur and the plasmid Gβ2m (38) by Dr. Kenneth Soprano after preparation from a 550 bp Pst I fragment of human β2-microglobulin kindly provided by Dr. K. Itakura, Harvard University.

Isolation and Purification of Plasmid DNA

Bacteria containing plasmids were grown in YTN medium containing 50 μg/ml ampicillin and amplification was induced in the presence of 200 μg/ml chloramphenicol. Plasmid DNA was isolated and purified using the following methods.

Rapid, Small Scale Plasmid DNA Isolation (80)

Small scale plasmid isolation was carried out using the rapid boiling method of Holmes and Quigley (80). An overnight culture (1.5 ml) of plasmid-containing bacteria was transferred to a microcentrifuge tube and centrifuged in an Eppendorf centrifuge at 12,000 X g for 2 minutes at room temperature. The supernatant was decanted and the pellet vortexed for 2 seconds and resuspended in 150 μl STET buffer (8% sucrose, 5% triton X-100, 50 mM EDTA, 50 mM Tris•Cl (pH 8.0)). After resuspension, 38 μl of freshly prepared

lysozyme (10 mg/ml) was added to the cell suspension, the mixture boiled for 60 seconds and centrifuged at 12,000 X g for 10 minutes at room temperature. The pellet was removed from the microcentrifuge tube using a toothpick and the DNA precipitated from the supernatant with 188 μl isopropanol at room temperature for 10 minutes. Following precipitation, the DNA was pelleted by centrifugation at 12,000 X g for 5 minutes at room temperature, the supernatant decanted and the pellet washed with 100 μl 70% ethanol at room temperature. The DNA pellet was dried under vacuum, resuspended in 40 μl 10 mM Tris•Cl, 1 mM EDTA (pH 8.0) containing DNase-free RNase (50 μg/ml) and incubated for 30 minutes at 37°C. Following incubation the DNA was stored at -20°C.

Large Scale Plasmid DNA Isolation

Large scale isolation of plasmid DNA was carried out using the alkaline lysis method described in Birnboim and Doly (11) and modified by D. Ish-Horowicz (87). A 500 ml culture of plasmid-containing bacteria was centrifuged at 5000 rpm in a Beckman JA10 rotor (4400 X g) for 15 minutes at 4°C. The supernatant was decanted and the cell pellet resuspended in 10 ml alkaline lysis solution #1 (see appendix B) containing 50 mg lysozyme and incubated for 5 minutes at room temperature. After the incubation, 20 ml of alkaline lysis solution #2 (see appendix B) was added,

the solution was mixed gently by inverting until no clumps remained and further incubated for 15 minutes at 4°C followed by the addition of 15 ml ice cold 5M potassium acetate (pH 4.8) and incubation at 4°C for an additional 15 minutes. Upon completion of the incubation the solution was transferred to a 50 ml polycarbonate centrifuge tube and centrifuged at 16,000 rpm in a Beckman JA20 rotor (31,000 X g) for 20 minutes at 4°C. The supernatant was transferred to a 150 ml corex centrifuge bottle containing 28 ml isopropanol, incubated at room temperature for 15 minutes and the DNA collected by centrifugation at 6000 rpm in a Beckman JA7.5 rotor (6700 X g) for 30 minutes at 20°C. After the centrifugation, the supernatant was decanted and the pellet washed with 10 ml 70% ethanol at room temperature. The DNA pellet was dried under vacuum and resuspended in 8 ml 10 mM Tris•Cl, 1 mM EDTA (pH 8.0).

The DNA collected after isopropanol precipitation was purified by CsCl buoyant density gradient centrifugation as described by Clewell and Helinski (33). Solid CsCl, 8 g, was added to the resuspended DNA (8 ml) and the solution was mixed gently until all of the salt was dissolved. A 10 mg/ml stock of ethidium bromide, 0.9 ml for every ml of DNA solution, was added and the final solution transferred to Beckman quick seal ultracentrifuge tubes. The plasmid DNA was banded by centrifugation at 53,000 rpm in a Beckman 70.1Ti ultracentrifuge rotor (270,000 X g) for 24 hours at

20°C. The band of supercoiled plasmid DNA was collected using an 18 gauge needle and the DNA passed over a cation exchange column of Dowex 50W-X8 followed by chromatography through a 30 X 1.5 cm BioGel A-15m column, developed with 10 mM Tris•Cl, 1 mM EDTA (pH 8.0). Fractions containing plasmid DNA (Vo) were pooled and ethanol precipitated. The purified DNA was quantitated and stored at either 4°C or -20°C. Alternatively, after collection of the supercoiled plasmid DNA band, the DNA solution was transferred to a dialysis sac and dialyzed sequentially for 6 hours, at 4°C, against 3 1000 X volumes of 10 mM Tris•Cl, 1 mM EDTA (pH 8.0). The purified DNA was then quantitated and stored at either 4°C or -20°C.

Isolation and Purification of Eukaryotic Genomic DNA

Eukaryotic genomic DNA was isolated and purified essentially as described by Gross-Bellard et al. (66) and modified by Blin and Stafford (14). Approximately 2 X 10^7 eukaryotic cells were collected by centrifugation at 2500 rpm in an IEC rotor (1400 X g) for 5 minutes at 4°C. The supernatant was decanted and the cells gently resuspended in cold PBS and recollected by centrifugation at 2500 rpm in an IEC rotor (1400 X g) for 5 minutes at 4°C. After centrifugation, the supernatant was decanted and the pellet was vortexed gently, resuspended in 10 ml DNA lysis solution (0.1 X SSC (20 X SSC: 3 M NaCl, 0.3 M sodium

citrate; pH 7.4), 0.5% SDS (w/v), 200 μg/ml proteinase K) and incubated for 24 hours at room temperature with very gently shaking. Upon completion of the incubation the solution was extracted with an equal volume of equilibrated phenol and centrifuged at 2500 rpm in an IEC rotor (1400 X g) for 10 minutes at room temperature. Next, the solution was extracted with an equal volume of $CHCl_3$/IAA (24:1, v/v) and an equal volume of equilibrated phenol and centrifuged at 2500 rpm in an IEC rotor (1400 X g) for 10 minutes at room temperature. This extraction was repeated until a clear interphase was visible. The final extraction was with an equal volume of $CHCl_3$/IAA (24:1, v/v) followed by centrifugation at 2500 rpm in an IEC rotor (1400 X g) for 10 minutes at room temperature. Upon completion of the extractions the aqueous phase was split into two fractions of equal volume and transferred to 30 ml corex centrifuge tubes. The DNA was precipitated with 0.5 ml 3 M sodium acetate and 15 ml 95% ethanol overnight at 4°C. The precipitated genomic DNA was hooked out with a bent glass rod, resuspended in 10 mM Tris•Cl, 1 mM EDTA (pH 8.0) and stored at 4°C. In addition, cellular DNAs used for chromosome localization were isolated as described (81,82).

Isolation and Purification of Mammalian RNA (131)

Isolation of total cellular mammalian RNA was carried out as previously described (131). Cells growing in

suspension culture were harvested by centrifugation at 2500 rpm in an IEC rotor (1400 X g) for 10 minutes at 4°C and washed twice with cold 1 X spinner salt solution (GIBCO). The cells were lysed in a solution (1.3 X 0^7 cells/ml lysis solution) containing 1.6 mM Tris•Cl (pH 7.4), 0.8 mM EDTA, 5.0 μg/ml PVS, 2.0% SDS (w/v) and 500 μg/ml proteinase K for 15-30 minutes at room temperature. Following the incubation, NaCl was added to 0.25 M and the aqueous phase was extracted once with an equal volume of a 1:1 mixture (v/v) of equilibrated phenol and $CHCl_3$/IAA (24:1, v/v) and twice with an equal volume of $CHCl_3$/IAA (24:1, v/v). Upon completion of the extractions the total nucleic acid was precipitated using 24 μl 5M sodium acetate (pH 5.5) and three volumes of 95% ethanol overnight at -20°C. The total nucleic acid was collected by centrifugation at 6000 rpm in a Beckman JA7.5 rotor (6700 X g) for 30 minutes at 20°C. After the centrifugation, the supernatant was decanted and the pellet washed with 10 ml 70% ethanol at room temperature. The pellet was dried under vacuum, resuspended in DNase I digestion buffer (20 mM Tris•Cl (pH 8.0), 10 mM $CaCl_2$) and incubated at 37°C for 20-40 minutes with 0.1 mg/ml DNase I (electrophoretically pure) which had been pretreated with proteinase K for 2 hours (174). Following the incubation, NaCl was added to 0.25 M, SDS to 2.0% (w/v) and extractions were carried out as described above. Upon completion of the extractions the RNA was precipitated using

a final concentration of 25 mM sodium acetate and three volumes of 95% ethanol overnight at -20°C. The total RNA was collected by centrifugation at 6000 rpm in a Beckman JA7.5 rotor (6700 X g) for 30 minutes at 20°C. Following the centrifugation, the supernatant was decanted and the pellet washed with 10 ml 70% ethanol at room temperature. The pellet was dried under vacuum, resuspended in ddH_2O, quantitated and stored at -20°C.

Selection of Poly A^+ RNA

Poly A^+ RNA was selected using a single pass over oligo dT-cellulose as described by Maniatis *et al.* (108). A single oligo dT-cellulose selection greatly enriches for poly A^+ RNA but does not totally remove all of the poly A^- RNA. Total cellular HeLa RNA in loading buffer (10 mM Tris-HCl (pH 7.6), 500 mM LiCl, 1.0 mM EDTA, 0.1% SDS (w/v)) was heated to 65°C for 5 minutes and applied to prewashed oligo dT-cellulose columns. The columns had been prewashed with i) three column volumes ddH_2O, ii) three column volumes 0.1 N NaOH, 5 mM EDTA and iii) three column volumes loading buffer. After addition of the sample to the column the flow-through was collected, heated to 65°C for 5 minutes, cooled and reapplied to the column. The column was then washed with 5 to 10 column volumes of loading buffer, followed by 4 column volumes of loading buffer containing 100 mM NaCl. The poly A^+ RNA was eluted with 2 to 3 column

volumes of elution buffer (10 mM Tris•Cl (pH 7.5), 1.0 mM EDTA, 0.05% SDS (w/v)) and precipitated with 300 mM sodium acetate (pH 5.2) and 2.2 volumes 95% ethanol overnight at -20°C. RNA was collected by centrifugation at 10,000 rpm in a Beckman JA20 rotor (12,000 X g) for 30 minutes at 20°C. Following the centrifugation, the supernatant was decanted and the pellet washed with 10 ml 70% ethanol at room temperature. The pellet was dried under vacuum, resuspended in ddH_2O, quantitated and stored at -20°C.

Spectrophotometric Quantitation of DNA and RNA

Nucleic acids, both DNA and RNA, were routinely quantitated by OD at 260 nm. An OD at 260 nm (OD_{260}) of 1 corresponds to approximately 50 μg/ml for double-stranded DNA, 40 μg/ml for single-stranded DNA and RNA, and 20 μg/ml for oligonucleotides (108). The purity of the nucleic acid was often assessed by calculating the 260 nm:280 nm optical density ratio (OD_{260}:OD_{280}). Pure preparations of DNA and RNA have an OD_{260}:OD_{280} of 1.8 and 2.0, respectively.

Preparation of Radiolabeled DNA

Random Oligonucleotide Primed Labeling

A random oligonucleotide priming technique (47,48,169), simplified by Roberts and Wilson (139), employing T7 DNA polymerase (57,168), was carried out using a random primer kit. The volume of a sample, containing 25 ng of DNA

template (linearized plasmid DNA or an isolated DNA fragment) to be labeled, was adjusted to 23 μl with ddH_2O. The solution was then heated in a boiling H_2O bath for 5 minutes and centrifuged briefly to collect the condensation. After heat denaturation, 10 μl of 5 X primer buffer (supplied by the manufacturer), 5 μl labeled nucleotide (~50 μci) ([α-^{32}P]dCTP (~3,000 Ci/mmole)) and 1 μl diluted T7 DNA polymerase (2 units/μl in enzyme dilution buffer supplied by the manufacturer) were added to the template DNA and the mixture was incubated at 37°C for 2-10 minutes. Upon completion of the incubation period, the reaction was quenched by the addition of 2 μl of stop mixture. The volume of the reaction mixture was adjusted to 1.2 ml with Elutip-d low salt buffer (200 mM NaCl, 20 mM Tris•Cl (pH 7.4), 1 mM EDTA) and the labeled DNA purified using a Schleicher & Schuell Elutip-d column as described by the manufacturer. The labeled DNA was stored at 4°C until use.

Labeling the 3' Termini of DNA (124)

DNA hybridization probes labeled at the 3' termini were prepared as described by O'Farrell (124). The volume of the solution containing the DNA fragment to be labeled was adjusted to 12 μl with ddH_2O. BSA, 2.5 μl of a 1 mg/ml stock, was added to the DNA solution along with 2.5 μl of Boehringer Mannheim restriction endonuclease 10 X digestion

buffer L and 2.5 μl T4 DNA polymerase (1 unit/μl) and the solution incubated for 2-5 minutes at 37°C. Immediately following the incubation, 1 μl of each of the following was added: 2 mM dGTP, 2 mM dATP, 2 mM TTP and 2 μl (~20 μci) of [α-^{32}P]dCTP (~3,000 Ci/mmole), and incubation was continued for 1 minute at 37°C. Upon completion of the incubation, 0.5 μl of 2 mM dCTP was added and the incubation continued for 10 minutes at 37°C. The reaction was stopped by heating the solution for 5 minutes at 70°C followed by the addition of 1 μl of 250 mM EDTA. The volume of the quenched reaction mixture was brought to 1.2 ml with Elutip-d low salt buffer and the labeled DNA purified using a Schleicher & Schuell Elutip-d column as described by the manufacturer. The labeled DNA was stored at 4°C until use.

Labeling the 5' Termini of DNA

DNAs to be labeled at the 5' termini and used as hybridization probes were treated with CIP as described by Chaconas and van de Sande (26) and modified by Maniatis et al. (108), followed by 5' end labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP (~3,000 Ci/mmole) as described by Maxam and Gilbert (114) and modified by Maniatis et al. (108). The volume of the solution containing the DNA fragment to be labeled was adjusted to 44 μl with ddH_2O. CIP 10 X buffer (500 mM Tris•Cl (pH 9.0), 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine), 5 μl, and 1 μl

of CIP (0.2 units/μl for DNA fragments with protruding 5' termini or 1 unit/μl for DNA fragments with blunt or recessed 5' termini) were added to the DNA solution and incubated for 15 minutes at 37°C followed by incubation for 15 minutes at 56°C. Upon completion of the 56°C incubation a second aliquot of CIP was added to the reaction mixture and the successive incubations were repeated. The reaction was quenched by the addition of 40 μl ddH_2O, 10 μl 10 X STE buffer (100 mM Tris•Cl (pH 8.0), 1 M NaCl, 10 mM EDTA) and 5 μl 10% SDS (w/v), and the CIP was heat inactivated for 30 minutes at 68°C. Upon completion of the incubation the reaction mixture was extracted twice with an equal volume of equilibrated phenol and twice with an equal volume of $CHCl_3$/IAA (24:1, v/v). Following the addition of 6.3 μl 5 M NaCl, 1 μl glycogen (20 mg/ml) and 330 μl 95% ethanol, the DNA was precipitated on crushed dry ice for 15 minutes and pelleted by centrifugation at 12,000 X g for 30 minutes at 4°C. After centrifugation, the supernatant was decanted and the pellet washed with 100 μl 70% ethanol at room temperature. The pellet of DNA was dried under vacuum, resuspended in 44 μl 10 mM Tris•Cl, 1 mM EDTA (pH 8.0) and stored at -20°C until the 5' end labeling reaction was carried out.

Spermidine, 1 μl of a 100 mM stock, and 5 μl 10 X kinase buffer (660 mM Tris•Cl (pH 9.5), 100 mM $MgCl_2$, 100 mM 2-mercapto-ethanol) were added to the solution of CIP

treated DNA and incubated for 5 minutes at 70°C. After the incubation, the solution was snap frozen in crushed dry ice. The frozen solution was quickly thawed, added to 10 μl (~100 μci) of [γ-^{32}P]ATP (~3,000 Ci/mmole) and incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 1 μl 100 mM EDTA and 1.2 ml Elutip-d low salt buffer, and the labeled DNA was purified using a Schleicher & Schuell Elutip-d column as described by the manufacturer. The labeled DNA was stored at 4°C until use.

Analysis of Recombinant DNA Clones

Mapping by Single and Multiple Restriction Endonuclease Digestions

Single and double digests were carried out on plasmid and λ bacteriophage DNAs using restriction endonucleases and analyzed electrophoretically on horizontal agarose gels (108).

Indirect End-Labeled Mapping

Restriction mapping of recombinant λ phage by indirect end-labelling was carried out as described by Rackwitz et al. (137) using hybridization conditions defined by Little and Cross (103). Oligonucleotides complementary to the cos sequence of λ phage were 5' end labeled using T4 polynucleotide kinase. Oligonucleotides (33 ng) in 1 μl were combined with 1 μl 10 X kinase buffer, 2.5 μl (~25 μci) of [γ-^{32}P]ATP (~3,000 Ci/mmole), 0.4 μl 100 mM spermidine,

3.8 μl ddH_2O and 1.5 μl of 10 unit/μl T4 polynucleotide kinase and the reaction mixture was incubated for 30 minutes at 37°C. The reaction was stopped by adding 0.4 μl 250 mM EDTA and 100 μl 10 mM Tris•Cl, 1 mM EDTA (pH 8.0) and heating for 10 minutes at 70°C. The reaction mixture was extracted with an equal volume of a 1:1 mixture (v/v) of equilibrated phenol and $CHCl_3$/IAA (24:1, v/v). Following the addition of 6.7 μl 5 M NaCl, 1 μl glycogen (20 mg/ml) and 350 μl 95% ethanol, the oligonucleotides were precipitated on crushed dry ice for 15 minutes and pelleted by centrifugation at 12,000 X g for 30 minutes at 4°C. After centrifugation, the supernatant was decanted and the pellet washed with 100 μl 70% ethanol at room temperature. The pellet was dried under vacuum, resuspended in 100 μl 10 mM Tris•Cl, 1 mM EDTA (pH 8.0) and stored at 4°C until the hybridization was carried out.

To 10 μl of partially restricted λ phage DNA (0.5-1.0 μg) was added 1 μl of a solution containing: 2% SDS (w/v), 50 mM EDTA, 1.0 μg/μl proteinase k and 0.026 ng of 5' end labeled oligonucleotides, and the mixture was incubated for 10 minutes at 75°C. Immediately following the incubation, the mixture was transferred to 45°C and hybridized for 60 minutes. Following the hybridization, the samples were placed on wet ice and 1 μl TPE tracking dye (36 mM Tris•Cl (pH 7.7), 30 mM NaH_2PO_4 (monobasic), 1 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol,

10% glycerol) was added. The samples were then loaded onto a 0.5% agarose TPE (36 mM Tris•Cl (pH 7.7), 30 mM NaH_2PO_4 (monobasic), 1 mM EDTA) gel and electrophoresed for 24-28 hours at 40 watts with buffer recirculation. Following electrophoresis, the gel was dried under vacuum for 1 hour at 80°C. Autoradiography was performed using pre-flashed Kodak XAR-5 X-ray film and a Cronex Lightning Plus screen at -70°C. I would like to acknowledge Dr. Paul L. Romain and Suzie Pilapil for their work on the mapping analysis of the λHHG5E genomic clone.

Southern Blot Analysis

Restriction endonuclease digested DNAs were separated by electrophoresis through agarose gels and transferred to Zeta-Probe blotting membrane as described by Reed and Mann (138). The agarose gel was soaked in 25 mM HCl for 10 minutes at room temperature, rinsed briefly in ddH_2O and the DNA was transferred with 0.4 N NaOH overnight at room temperature. Following transfer, the membrane was baked in a vacuum oven at 80°C for 1 hour, prewashed in 0.5% SDS (w/v), 0.1 X SSC for one hour at 65°C and then prehybridized and hybridized using a modification of the method described by Wahl *et al.* (180). Prehybridization was carried out for two hours in 50 mM sodium phosphate (pH 6.5), 0.45% SDS (w/v), 47% formamide, 9% dextran sulfate and 500 μg/ml double stranded *E. coli* DNA. After prehybridization,

^{32}P-labeled probe was added to the mix to a concentration of 1 X 10^6 dpm/ml and hybridized for 6-18 hours at 48°C. The hybridized membrane was then washed three times as follows: 1) in 100 ml 100 mM potassium phosphate (pH 7.4) for 30 minutes at room temperature; 2) in 100 ml 100 mM potassium phosphate (pH 7.4) for 30 minutes at 60°C; 3) in 100 ml 1 X SSC and 0.2% SDS (w/v) for 30 minutes at 60°C. Autoradiography was performed using pre-flashed Kodak XAR-5 X-ray film and a Cronex Lightning Plus screen at -70°C. ^{32}P-labeled probes used for Southern analysis of the genomic H2B clone λHHG5E were as follows: H1, 1445 nt Pst I/Eco RI fragment from pFNC16 (131); H2A, 980 nt Sst I fragment from pFF435 (112); H2B, 340 nt Eco RI/Xho I fragment from λHHC289 (Figure 3-6); H3, 2100 nt Eco RI fragment from pST519 (111); H4, 1740 nt Hind III/Eco RI fragment from pFO108A (131).

Recovery of DNA Fractionated Electrophoretically

An agarose slice containing the DNA fragment of interest was cut into very fine pieces, using a clean scalpel, and the resulting agarose puree was then placed into the pocket formed by folding a large piece of parafilm into quarters. The puree was frozen on crushed dry ice followed by thawing while applying pressure with the thumb and forefinger. The resulting liquid which was forced out of the puree was drawn off with a pipette and placed into a clean tube. This process, including the freezing step, was

repeated until no additional liquid was squeezed out of the puree. The DNA fragment was purified using a Schleicher & Schuell Elutip-d column as described by the manufacturer and was stored at -20°C.

Analysis of Mammalian RNA

Agarose-Formaldehyde Denaturing Gel Electrophoresis

RNA was size fractionated in a 1.5% agarose, 1 X MOPS (20 mM MOPS (pH 7.0), 5 mM sodium acetate, 1 mM EDTA), 6% formaldehyde gel using a 1 X MOPS, 3.7% (v/v) formaldehyde running buffer, a modification of the method used by Lehrach et al. (99).

Northern Analysis

Northern blot analysis was carried out as follows: the RNA was size fractionated as described above and transferred as described by Thomas (170) to Zeta-Probe blotting membrane using 20 X SSC as the transfer buffer. Following transfer, the membrane was baked in a vacuum oven at 80°C for 1 hour, prewashed in 0.5% SDS (w/v), 0.1 X SSC for one hour at 65°C and then prehybridized and hybridized using a modification of the method described by Wahl et al. (180). Prehybridization was carried out for two hours in 50 mM sodium phosphate (pH 6.5), 0.45% SDS (w/v), 47% formamide, 9% dextran sulfate and 500 μg/ml double stranded E. coli DNA. After prehybridization, ^{32}P-labeled probe was added to

the mix to a concentration of 1 X 10^6 dpm/ml and hybridized for 6-18 hours (the temperature of the hybridization was dependent upon probe size and percent GC content and ranged from 38°C-48°C). The hybridized Zeta-Probe membrane was then washed five times as follows: 1) 10 minutes at room temperature with 5 X SSC, 1 X Denhardt's; 2) 30 minutes at 60°C with 5 X SSC, 1 X Denhardt's; 3) 30 minutes at 60°C with 2 X SSC, 0.1% SDS (w/v); 4) 30 minutes at 60°C with 1 X SSC, 0.1% SDS (w/v); 5) 30 minutes at 60°C with 0.1 X SSC, 0.1% SDS (w/v). Autoradiography was performed using pre-flashed Kodak XAR-5 X-ray film and a Cronex Lightning Plus screen at -70°C. The developed X-ray films were analyzed by laser densitometry using a LKB 2400 GelScan XL densitometer. β-particle analysis of hybridized membranes was carried out using a βetagen (Waltham, MA) Betascope 603 blot analyzer.

S1 Nuclease Protection Analysis

S1 analysis was carried out according to Berk and Sharp (6) as modified by Haegeman et al. (67). DNA fragments used as probes (preparation described above) were treated with CIP as described by Chaconas and van de Sande (26) and modified by Maniatis et al. (108) followed by 5' end labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP (~3,000 Ci/mmole) as described by Maxam and Gilbert (114) and modified by Maniatis et al. (108). Alternatively, DNA

fragments were 3' end labeled with T4 DNA polymerase and [α-^{32}P]dCTP (~3,000 Ci/mmole) as described by O'Farrell (124). The RNA to be analyzed was co-precipitated with labeled DNA probe using 0.3 M NaCl, 1.0 μl glycogen (20 mg/ml) and 2.5 volumes of 95% ethanol for 15 minutes on crushed dry ice and pelleted by centrifugation at 12,000 X g for 30 minutes at 4°C. After centrifugation, the supernatant was decanted and the pellet washed with 100 μl 70% ethanol at room temperature. The pellet was dried under vacuum, resuspended in 5 μl 5 X S1 hybridization buffer (5 X: 2 M NaCl, 200 mM Pipes (pH 6.4), 5 mM EDTA) and 20 μl deionized, recrystallized formamide. Hybridization mixtures, 25 μl, were heated at 90°C for 10 minutes and then annealed at 55°C for at least three hrs. Annealing reactions were quenched with eight volumes, 200 μl, S1 nuclease buffer (25 mM NaCl, 30 mM sodium acetate (pH 4.6), 1.0 mM $ZnSO_4$) followed by incubation at 37°C for 30 minutes with 900 units of S1 nuclease in the presence of 20 μg salmon sperm DNA. After S1 digestion the samples were extracted with an equal volume of a 1:1 mixture of equilibrated phenol and $CHCl_3$/IAA (24:1, v/v) and ethanol precipitated using 0.3 M NaCl, 1.0 μl glycogen (20 mg/ml) and 2.5 volumes of 95% ethanol overnight at -20°C. The protected fragment was pelleted by centrifugation at 12,000 X g for 30 minutes at 4°C. Following centrifugation, the supernatant was decanted and the pellets were washed

with 100 μl 70% ethanol at room temperature. The pellets were dried under vacuum, resuspended in 6 μl loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) and the samples were heat denatured and loaded onto a 6% polyacrylamide sequencing gel containing 7.8 M urea (108). After electrophoresis the gel was soaked in 10% acetic acid, 12% methanol for 15-30 minutes to remove urea, dried under vacuum at 80°C, and placed against preflashed Kodak XAR-5 X-ray film and a Cronex Lightning Plus screen at -70°C.

In Vitro Nuclear Run-on Transcription Analysis

The in vitro nuclear run-on transcription assays were performed using a modification (4) of the method used by Flint et al. (51). Cells were harvested by centrifugation and the cell pellets washed twice in cold isotonic buffer (125 mM potassium phosphate, 30 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol). Cells were disrupted by homogenization (6-12 strokes) with a Dounce homogenizer, Wheaton type A pestle. After >90% of the cells had been lysed, nuclei were pelleted by centrifugation at 2000 rpm for 10 minutes in an IEC centrifuge at 4°C and resuspended in nuclei storage buffer (50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.1 mM EDTA) containing 40% glycerol. Nuclei were aliquoted and used fresh in the in vitro transcription reactions. Reactions typically contained 10^7 nuclei,

100 μCi α-^{32}P-UTP (3000 Ci/mmole), 1 mM ATP, 0.25 mM GTP and 0.25 mM CTP in a final volume of 130 μl and were incubated for 30 minutes with intermittent shaking at 30°C. Radiolabeled RNAs were isolated by treatment of nuclei with DNase I (100 μg/ml) in the presence of 0.6 M NaCl, 50 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$ for 15 minutes at room temperature. The mixture was then incubated with proteinase K (200 μg/ml) for 30-60 minutes at 37°C in the presence of 150 mM NaCl, 12.5 mM EDTA, 100 mM Tris-HCl (pH 7.5) and 20 mM $MgCl_2$. Sodium acetate (pH 5.5) was added to 200 mM and nucleic acids were extracted several times by the hot phenol method (32,148). To the aqueous solution of ^{32}P-labeled RNAs, 150 μg of yeast RNA and 2.5 volumes of 95% ethanol were added. Precipitation was overnight at -20°C. Radiolabeled transcripts were resuspended in 1 ml of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and an aliquot of each sample was precipitated with 150 μg yeast RNA and cold 10% TCA. TCA-precipitable counts were determined by liquid scintillation spectrometry.

Following preparation of Southern blots (157) of electrophoretically-separated restriction endonuclease-digested plasmid DNAs or slot blots (prepared using a slot blot apparatus with conditions as described by Schleicher and Schuell Inc.) of linearized plasmid DNAs, DNA excess hybridizations were carried out. Southern or slot blots on nitrocellulose were prehybridized in 1 M NaCl, 20 mM

Tris-HCl (pH 7.4), 2 mM EDTA, 0.1% SDS (w/v), 5 X Denhardt's, 25 μg/ml denatured E. coli DNA and 12.5 mM sodium pyrophosphate at 65°C for at least 6 hours. Hybridizations were conducted at 65°C for 72 hours in 1 M NaCl, 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.1% SDS (w/v), 2.5 X Denhardt's, 25 μg/ml E. coli DNA with ^{32}P-labeled transcripts at 5 X 10^5-1 X 10^6 TCA-precipitable dpm/ml of hybridization solution. Blots were washed at 65°C for 15 minutes in fresh prehybridization solution, 1 hour in 2 X SSC, 0.1% SDS (w/v), overnight in 2 X SSC, 0.1% SDS (w/v) and 1 hour in 0.2 X SSC, 0.1% SDS (w/v). Autoradiography was performed on air dried filters with pre-flashed XAR-5 or Cronex film and Cronex Lightning Plus screens at -70°C. The developed X-ray films were analyzed by laser densitometry using a LKB 2400 GelScan XL densitometer. I would like to acknowledge Anna Ramsey-Ewing for her work on the transcriptional analysis of the H2B-GL105 gene.

Library Screening

Lambda gt11 cDNA Library Screening

A λgt11 human liver cDNA library, made from total cellular poly A$^+$ RNA and generously provided by Dr. S. L. C. Woo (Baylor College of Medicine, Department of Cell Biology), was screened with the ^{32}P-labeled human histone probes indicated in Figure 2-1. The library screening was

carried out as indicated in Figure 2-2 according to the procedure described by Benton and Davis (5) with the following modifications in the hybridization conditions. The filters were prehybridized at 60°C for two hours in a solution containing 5 X Denhardt's (100 X: 2% ficoll, 2% polyvinyl-pyrrolidone), 5 X SSPE (20 X: 3.6 M sodium chloride, 200 mM sodium phosphate, 20 mM EDTA; pH 7.4), 0.2% SDS (w/v), 0.1% w/v BSA and 500 μg/ml double stranded E. coli DNA. Hybridization was carried out at 60°C for 24 hours in a solution containing 0.5 X Denhardt's, 5 X SSPE, 0.2% SDS (w/v), 0.01% w/v BSA, 500 μg/ml E. coli DNA and 2 X 10^6 dpm/ml heat-denatured probe DNA fragment. Hybridized filters were then washed at 24°C for 30 minutes in 100 mM potassium phosphate (pH 7.4) followed by two 30 minute washes at 60°C in 2 X SSPE, 0.3% SDS (w/v). Autoradiography was performed using Kodak XAR-5 X-ray film and a Cronex Lightning Plus screen at -70°C.

Plaque dot analysis was carried out according to the procedure described by Powell et al. (132). A 100 μl aliquot of plating bacteria was placed into a tube and 4 ml of NZCYM top agar was added to the tube, vortexed gently and poured onto a NZCYM bottom agar plate. Upon solidification

Figure 2-1. Schematic diagram of histone gene probes used for the λgt11 poly A+ CDNA library screening

A λgt11 human liver cDNA library was screened with the ^{32}P-labeled human histone probes A, B, C, D, and E indicated by the dotted lines, set apart by arrows, beneath each restriction map. The human histone probes are as follows: A, 2100 nt Eco RI H3 fragment; B, 1740 nt Hind III/Eco RI H4 fragment; C, 550 nt Sst I H2B fragment; D, 980 nt Sst I H2A fragment; E, 1445 nt Pst I/Eco RI H1 fragment. λHHG41, λHHG55 and λHHG415 are λ Charon 4A recombinant bacteriophage which were previously isolated (24,154) from a λ Charon 4A genomic DNA library.

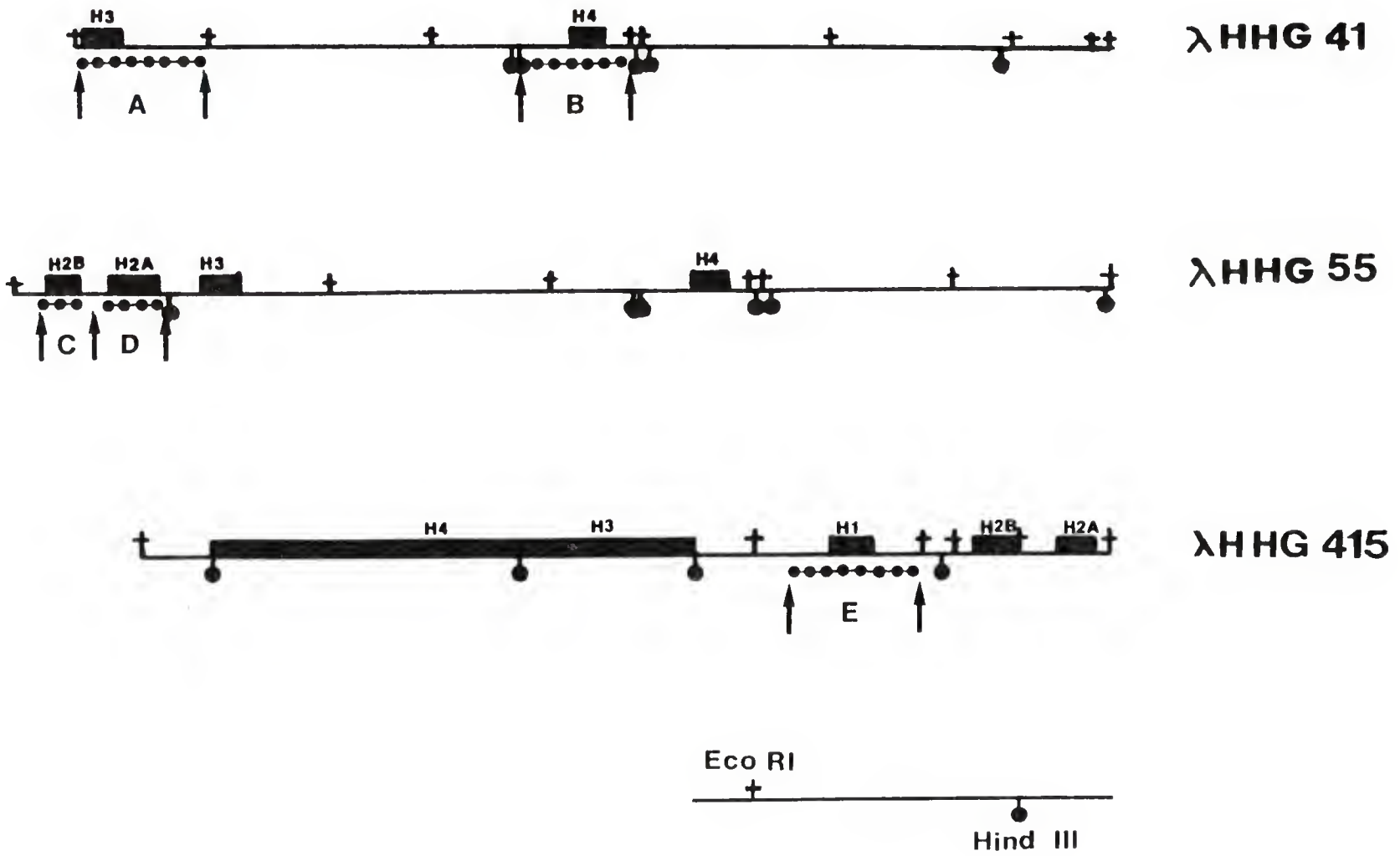
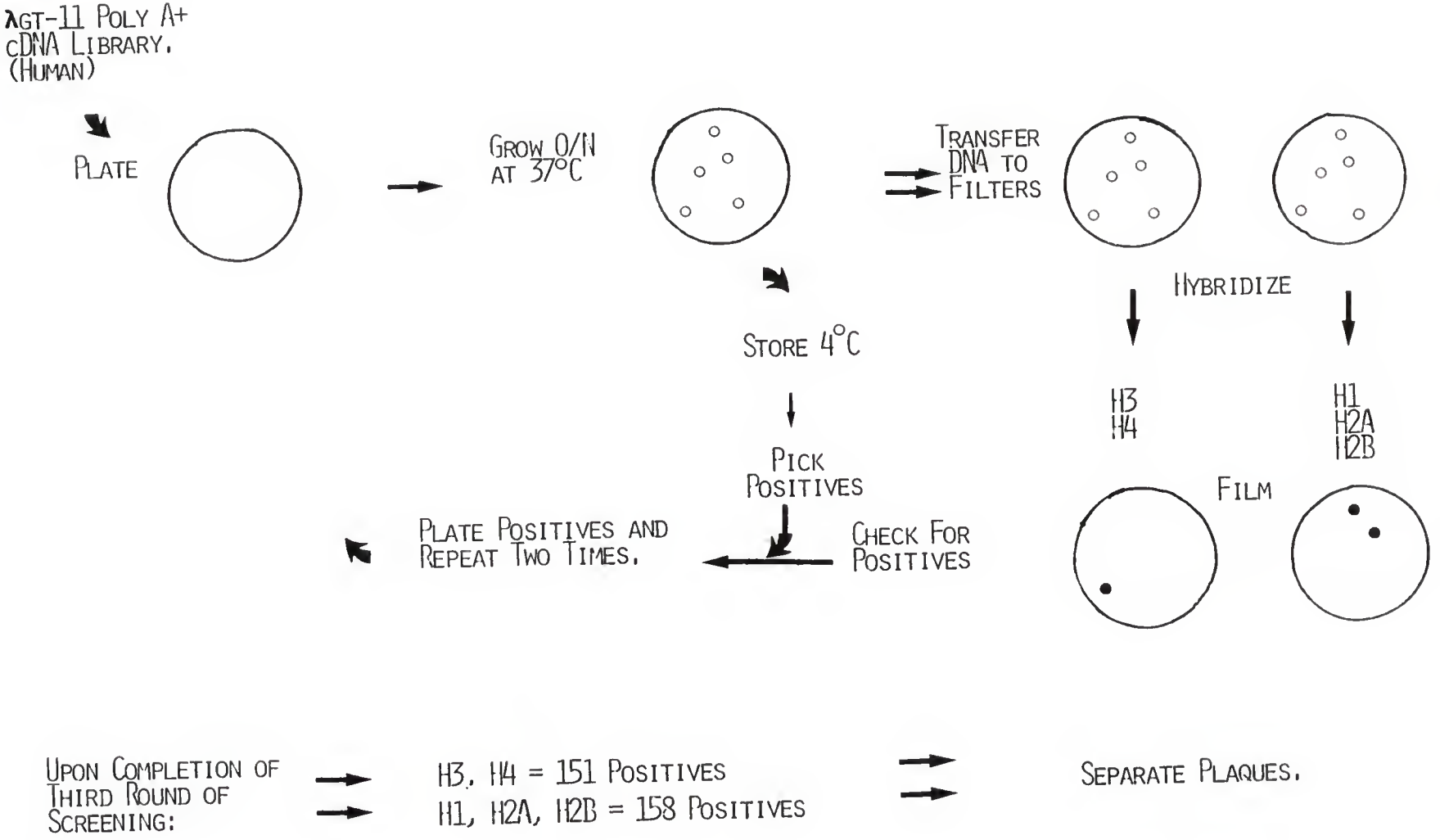

H3
H4
A
B
λHHG 41
H2B
H2A
H3
H4
C
D
λHHG 55
H4
H3
H1
H2B
H2A
E
λHHG 415
Eco RI
Hind III

Figure 2-2. Outline of the screening scheme for the λgt11 poly A+ cDNA library

A λgt11 human liver cDNA library was screened as described by Benton and Davis (5). The λgt11 cDNA library was grown on 100 mm diameter petri dishes. Following overnight incubation, duplicate nitrocellulose filters were lifted from each plate and prepared as described (5). One set of filters was hybridized to the H3 and H4 probe at 60°C and the other set to the H2A, H2B and H1 probes at 58°C as described by Lawn et al. (97). Hybridized filters were then washed and autoradiography performed. The positive plaques (or plaque areas) were picked and screened a second time. Single well isolated positive plaques were picked and screened a third time. Upon completion of the third round of screening 151 H3/H4 positives and 158 H2A/H2B/H1 positive plaques had been obtained. Positive plaques were separated according to individual histone class, using the human histone probes described in Figure 2-1, by plaque dot analysis.


λGT-11 Poly A+
cDNA Library.
(Human)
Plate
Grow O/N
at 37°C
Transfer
DNA to
Filters
Hybridize
Store 4°C
H3
H4
H1
H2A
H2B
Pick
Positives
Film
Plate Positives and
Repeat Two Times.
Check For
Positives
Upon Completion of
Third Round of
Screening:
H3, H4 = 151 Positives
H1, H2A, H2B = 158 Positives
Separate Plaques.

of the top agar, 0.5 μl aliquots of λ bacteriophage dilutions were spotted onto the plate and allowed to dry. The plate was inverted and incubated at 37°C for 5-12 hours to allow plaque formation. Up to 3 nitrocellulose filter lifts were prepared from the plate and hybridized to various probes as described in the previous section.

Lambda EMBL4 Genomic DNA Library Screening

An adult human lymphocyte genomic library, containing inserts of between 15 and 20 Kb, was prepared by inserting Sau3A I partially digested adult human lymphocyte genomic DNA into the Bam HI site of λEMBL4 and was generously provided by Dr. Paul Dobner (University of Massachusetts Medical Center Department of Molecular Genetics and Microbiology). A complete equivalent of this λEMBL4 human genomic DNA library (8 X 10^5 phage) was grown on 14 150 mm diameter petri dishes and screened with a ^{32}P-labeled H2B 3' non-translated probe (900 nt Sac I fragment isolated from λHHC289) according to the procedure described by Benton and Davis (5). LE392 (121) plating bacteria, 0.3 ml, were mixed with 50,000 pfu of recombinant phage and incubated for 20 minutes at 37°C. Next, 6.5 ml melted top agarose (46°C) was added and the mixture poured onto a 150 mm NZCYM bottom agar plate. The top agarose was allowed to solidify at room temperature and then incubated 10-12 hours (Or until the plaques reach a diameter of approximately 1.5 mm) at 37°C.

Following overnight incubation the plates were chilled for 60 minutes at 4°C. For filter lifting, plates were removed from the cold box and nitrocellulose filters carefully laid on top of the agarose for 2-3 minutes. The orientation of the filters was marked in three asymmetric positions using a nitrocellulose marking pen. The filters were then removed and soaked in denaturing solution (1.5 M NaCl, 0.5 N NaOH) for 30-60 seconds and transferred into neutralizing solution (1.5 M NaCl, 0.5 M Tris•Cl (pH 8.0)) for 5 minutes. Subsequently the filters were rinsed in 2 X SSPE, blotted dry on Whatman 3 MM paper and baked for 2 hours at 80°C in a vacuum oven. Hybridization of these filters was carried out essentially as described by Lawn et al. (97) with the following modifications in the hybridization conditions. The filters were prehybridized at 60°C for two hours in a solution containing 5 X Denhardt's, 5 X SSPE, 0.2% SDS (w/v), 0.1% w/v BSA and 500 μg/ml double stranded E. coli DNA. Hybridization was carried out at 60°C for 24 hours in a solution containing 0.5 X Denhardt's, 5 X SSPE, 0.2% SDS (w/v), 0.01% BSA, 500 μg/ml E. coli DNA and 2 X 10^6 dpm/ml heat-denatured probe DNA fragment. Hybridized filters were then washed (10 ml wash solution/filter) at 24°C for 0.5 hour in 100 mM potassium phosphate (pH 7.4) followed by two 0.5 hour washes at 60°C in 2 X SSPE, 0.3% SDS (w/v). Autoradiography was performed using Kodak XAR-5 X-ray film and a Cronex Lightning Plus screen at -70°C. Following

hybridization, the positive plaques (or plaque areas) were picked and placed into 1 ml of SM containing a drop of chloroform. These positive plaques were screened a second time by hybridization. Single, well isolated, positive plaques were picked and screened a third time.

Cloning and Construct Preparation

Subcloning into Plasmid Vectors

DNA fragments were isolated from agarose gels, and ligations into the appropriate vectors were carried out using T4 DNA ligase as described by the manufacturer. The ligation products were used to transfect competent E. coli DH5α (8) (see appendix B) bacteria. The 6100 nt Pst I fragment, 4100 nt Eco RI fragment and 6100 nt Eco RI fragment from λHHG5E (Figure 4-7) were isolated and cloned into pUC19 and the resulting subclones termed pGL105, pGL101 and pGL102, respectively.

Construct Preparation

Construct I (pGL105SV) is the control construct containing the entire λHHG5E H2B gene, promoter and 2000 nt of 3' untranslated region (Figure 2-3). The 6200 nt Pst I fragment from λHHG5E was isolated and ligated, using T4 DNA ligase, into the Pst I site of pUC19 to obtain the intermediate (pGL105). Construct I was obtained by isolating the Eco RI/Eco RI SV40 enhancer element from

pSVE108A (153), blunt-ending its termini with the Klenow fragment of DNA polymerase I and ligating it into the Bam HI site of pGL105 which had previously been blunt-ended with the Klenow fragment.

Construct II (pGL110SV) is similar to construct I but has the 3' Eco RI/Pst I fragment, containing the poly(A) addition sequences, deleted (Figure 2-4). The 4100 nt Eco RI fragment from λHHG5E was isolated, blunt-ended using the Klenow fragment and ligated using T4 DNA ligase into the Hinc II site of pUC8SV40E, which contains the SV40 enhancer element from pSVE108A (153), resulting in pGL110SV.

Construct III (pGL109SV) is similar to construct I but has the 170 nt Xho I fragment, containing the histone 3' hairpin motif, deleted (Figure 2-5). Preparation of construct III was carried out as follows. The intermediate pGL106 was prepared by isolating the 4200 nt Hind III fragment from pGL105 and ligating it, using T4 DNA ligase, into the Hind III site of pUC19. Next, the intermediate pGL107 was prepared by digesting pGL106 with Xho I to remove the 170 nt Xho I fragment containing the hairpin motif region and recircularizing the remaining fragment using T4 DNA ligase. The final construct was obtained by isolating the 4200 nt Hind III fragment from pGL107 and ligating it into the 4300 nt Hind III fragment from pGL105SV to give pGL109SV.

Figure 2-3. Outline of the cloning scheme for the construction of the H2B construct pGL105SV

Construct I (pGL105SV) is the control construct containing the entire λHHG5E H2B gene, promoter and 2000 nt of 3' untranslated region (Figure 2-3). The 6200 nt Pst I fragment from λHHG5E was isolated and ligated, using T4 DNA ligase, into the Pst I site of pUC19 to obtain the intermediate (pGL105). Construct I was obtained by isolating the Eco RI/Eco RI SV40 enhancer element from pSVE108A (153), blunt-ending its termini with the Klenow fragment of DNA polymerase I and ligating it into the Bam HI site of pGL105 which had previously been blunt-ended with the Klenow fragment. Restriction endonuclease sites are as follows: A, Asp 718; B, Bam HI; E, Eco RI; H, Hind III; P, Pst I; S, Sac I; S', Sph I; X, Xho I; a letter containing a semicircle hat indicates a restriction endonuclease site which has been destroyed due to a blunt-ending reaction. λHHG5E is a λEMBL4 recombinant bacteriophage which was isolated from a λEMBL4 genomic DNA library (see chapter 4).

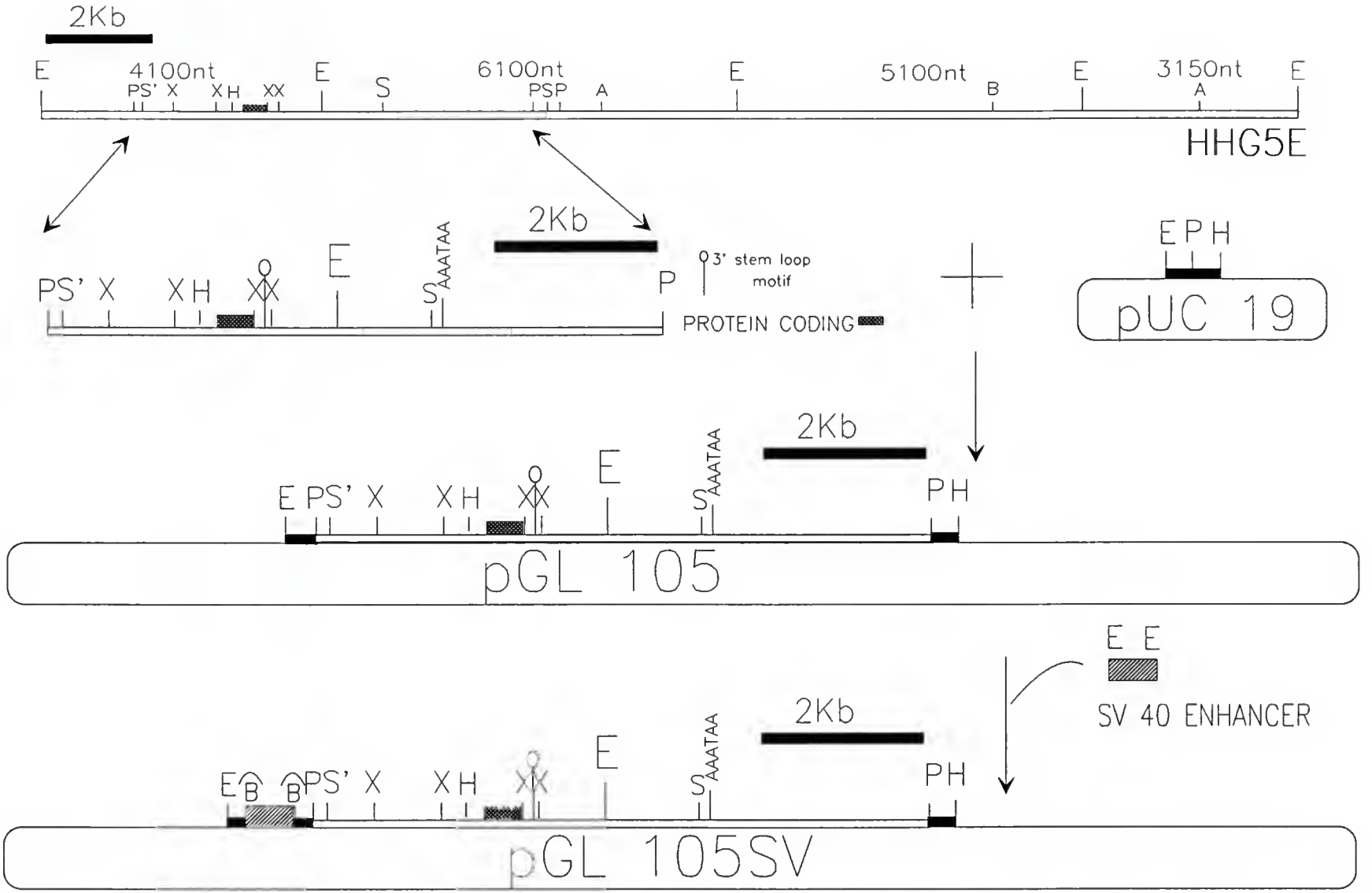

2Kb
E
4100nt
PS' X XH XX
E
S
6100nt
PSP
A
E
5100nt
B
E
3150nt
A
E
HHG5E
2Kb
PS' X XH XX
E
AAATAA
S
P
3' stem loop motif
PROTEIN CODING
E P H
pUC 19
2Kb
E PS' X XH XX
E
AAATAA
S
P H
pGL 105
E E
SV 40 ENHANCER
2Kb
E B B PS' X XH XX
E
AAATAA
S
P H
pGL 105SV

Figure 2-4. Outline of the cloning scheme for the construction of the H2B 3' deletion construct pGL110SV

Construct II (pGL110SV) is similar to construct I but has the 3' Eco RI/Pst I fragment, containing the poly(A) addition sequences, deleted (Figure 2-4). The 4100 nt Eco RI fragment from λHHG5E was isolated, blunt-ended using the Klenow fragment and ligated using T4 DNA ligase into the Hinc II site of pUC8SV40E, which contains the SV40 enhancer element from pSVE108A (153), resulting in pGL110SV. Restriction endonuclease sites are as follows: A, Asp 718; B, Bam HI; E, Eco RI; H, Hind III; P, Pst I; S, Sac I; S', Sph I; X, Xho I; a letter containing a semicircle hat indicates a restriction endonuclease site which has been destroyed due to a blunt-ending reaction. λHHG5E is a λEMBL4 recombinant bacteriophage which was isolated from a λEMBL4 genomic DNA library (see chapter 4).

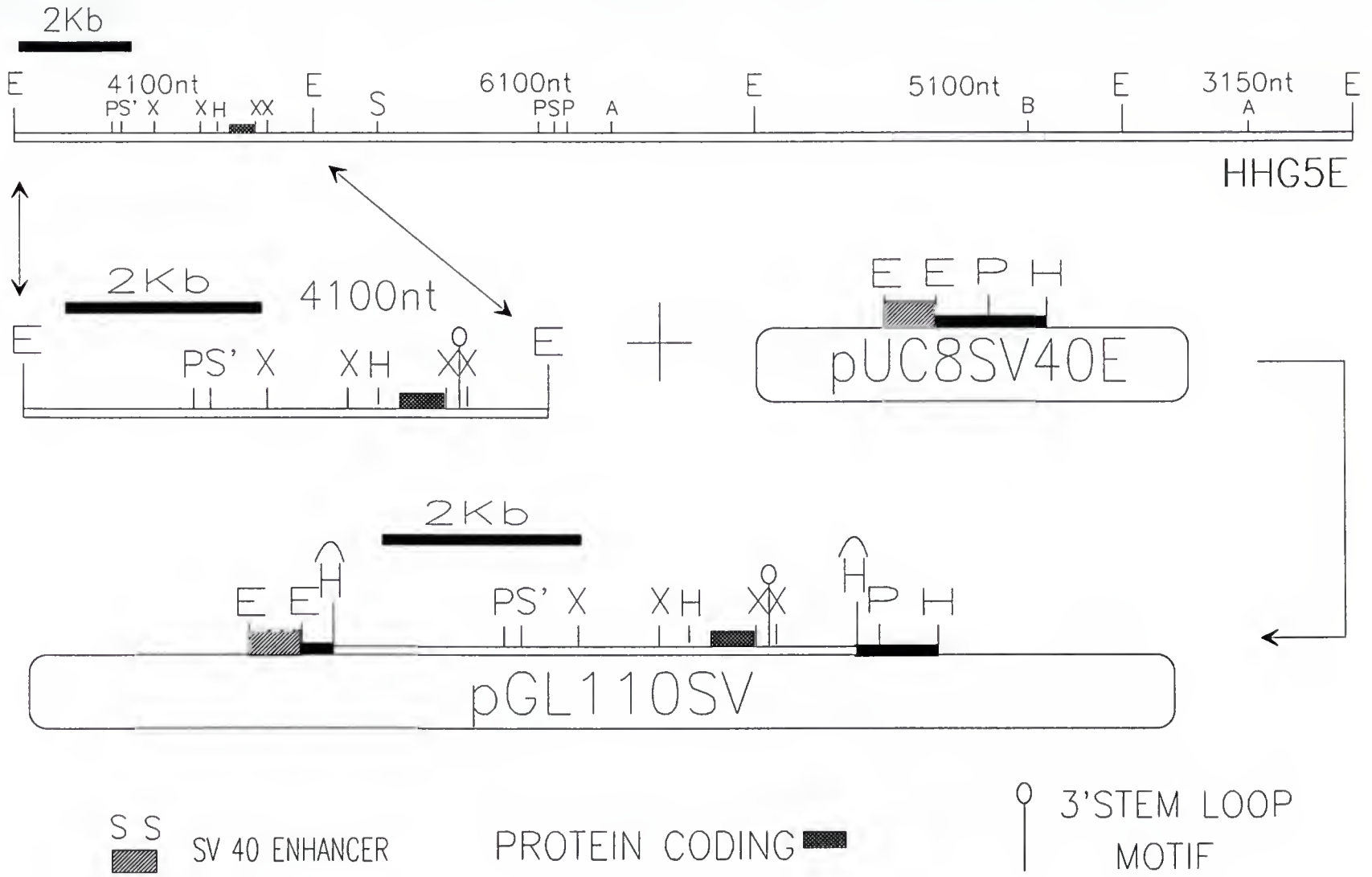
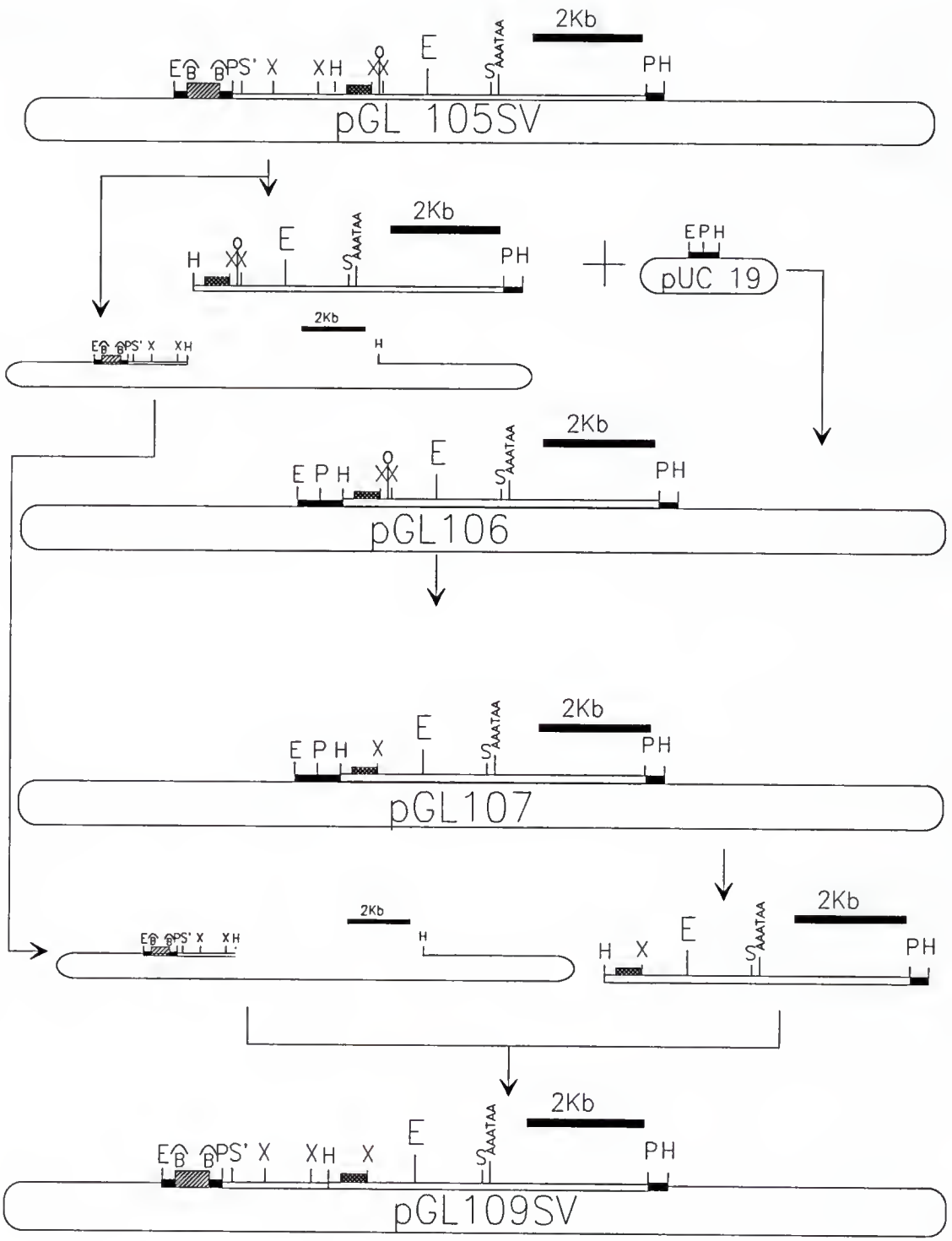

2Kb
E
4100nt
PS' X
XH
XX
E
S
6100nt
PSP
A
E
5100nt
B
E
3150nt
A
E
HHG5E
2Kb
4100nt
E
PS' X
XH
E
E E P H
pUC8SV40E
2Kb
E E H
PS' X
XH
H
P H
pGL110SV
S S
SV 40 ENHANCER
PROTEIN CODING
3'STEM LOOP
MOTIF

Figure 2-5. Outline of the cloning scheme for the construction of the H2B hairpin motif minus construct pGL109SV

Construct III (pGL109SV) is similar to construct I but has the 170 nt Xho I fragment, containing the histone 3' hairpin motif, deleted (Figure 2-5). Preparation of construct III was carried out as follows. The intermediate pGL106 was prepared by isolating the 4200 nt Hind III fragment from pGL105 and ligating it, using T4 DNA ligase, into the Hind III site of pUC19. Next, the intermediate pGL107 was prepared by digesting pGL106 with Xho I to remove the 170 nt Xho I fragment containing the hairpin motif region and recircularizing the remaining fragment using T4 DNA ligase. The final construct was obtained by isolating the 4200 nt Hind III fragment from pGL107 and ligating it into the 4300 nt Hind III fragment from pGL105SV to give pGL109SV. Restriction endonuclease sites are as follows: B, Bam HI; E, Eco RI; H, Hind III; P, Pst I; S, Sac I; S', Sph I; X, Xho I; a letter containing a semicircle hat indicates a restriction endonuclease site which has been destroyed due to a blunt-ending reaction. Plasmid GL105SV is described in Figure 2-3.


2Kb
E
AAATAA
PH
pGL 105SV
pUC 19
pGL106
pGL107
pGL109SV

Subcloning into Bacteriophage M13 Vectors

DNA fragments to be sequenced were isolated from agarose gels and ligated into M13mp18 and/or mp19 replicative form (123,190) using T4 DNA ligase as described by the manufacturer. The ligation products were used to transfect competent XL1-Blue (21) (see appendix C) bacteria.

Transfection of DNA into Bacterial Cells

Preparation of Competent E. coli Cells for Transfection

Competent E. coli cells were prepared using a modification of the method described by Mandel and Higa (107). An overnight culture of E. coli was diluted 1:100 into 100 ml YTN medium and grown at 37°C with vigorous shaking until the OD at 590 nm reached approximately 0.37. The cell culture was chilled on wet ice for 5 minutes, split into two 50 ml tubes and then centrifuged at 2500 rpm in an IEC rotor (1400 X g) for 10 minutes at 4°C. Following centrifugation the supernatant was discarded and the cell pellets vortexed gently, resuspended in 10 ml cold sterile transfection buffer (60 mM $CaCl_2$, 10 mM Pipes (pH 7.0), 15% glycerol) and centrifuged at 2500 rpm in an IEC rotor (1400 X g) for 10 minutes at 4°C. Next the supernatant was discarded and the cell pellets were gently resuspended in 10 ml cold sterile transfection buffer and incubated for 30 minutes on wet ice. Upon completion of the incubation the cells were centrifuged at 2500 rpm in an IEC rotor

(1400 X g) for 10 minutes at 4°C, the supernatant was discarded, and the cell pellets were gently resuspended in 2.5 ml cold, sterile transfection buffer. The competent cells were stored at 4°C and used within 24 hours of preparation.

Transfection of Recombinant Plasmid DNA

Transfection of recombinant plasmid DNA was carried out using a modification of the method described by Mandel and Higa (107). A fraction of a ligation mixture (1/10-1/2) and 100 μl competent E. coli DH5α (8) cells were combined in a 1.5 ml microcentrifuge tube, incubated for 10 minutes on wet ice and then incubated for 5 minutes at 37°C. Following the incubation the cells were diluted with 1.0 ml prewarmed YTN medium and incubated with shaking for 1 hour at 37°C. An aliquot of the transfection mixture was plated onto the appropriate bottom agar plate and incubated inverted for 12-18 hours at 37°C.

Transfection of Recombinant Bacteriophage M13 DNA

Transfection of recombinant bacteriophage M13 DNA was carried out as described by Messing (115). A fraction of a ligation mixture (1/10-1/2) and 200 μl competent E. coli XL1-Blue (21) cells were combined in a 14 mm X 100 mm glass culture tube, incubated for 40 minutes on wet ice and then heat-shocked for 2 minutes at 42°C. Immediately following

the incubation, 4 ml of YTN top agar (45°C) was added to the tube, vortexed gently and poured onto a NZCYM bottom agar plate. Upon solidification of the top agar the plate was incubated inverted for 12-18 hours at 37°C to allow for plaque formation.

DNA Sequencing

Sanger Dideoxy-Mediated Chain Termination Method

Dideoxy sequencing (146) was carried out using a Sequenase 2.0 DNA sequencing kit according to conditions described by the manufacturer. After the sequencing reactions were completed, loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) was added and the samples were heat denatured at 72°C for 2 minutes and loaded onto a 6% polyacrylamide sequencing gel containing 7.8 M urea (108). Following electrophoresis the gel was soaked in 10% acetic acid, 12% methanol for 15-30 minutes to remove urea, dried under vacuum at 80°C using 3 sheets of Whatman 3MM paper as backing, and placed against preflashed Kodak XAR-5 or Cronex X-ray film at room temperature.

Chromosomal in situ Hybridization

Chromosomal *in situ* hybridization was performed as described (23). Probe DNA (871 nt *Eco* RI/*Sac* I fragment isolated from λHHC289 (35)) was labeled with ^{3}H to a

specific activity of 2-4 X 10^7 dpm/μg. Slides containing metaphase chromosomes from normal male (46 XY) peripheral blood lymphocytes were aged at 4°C for 7-10 days and pretreated with ribonuclease A (Sigma) for 1 hour at 37°C. The chromosomal DNA was denatured at 70°C for 2 minutes in a 70% formamide: 2 X SSC mixture (pH 7.0). The probe DNA was denatured in a hybridization mixture containing 50% formamide, 2 X SSC and 10% dextran sulfate (pH 7.0). Hybridization was carried out at 37°C overnight. After rinsing at 39°C in 3 changes of 50% formamide: 2 X SSC and 5 changes of 2 X SSC, slides were dehydrated, air dried, subjected to autoradiography and banded with Wright's-Giemsa stain solution mixed with 1-3 parts of a pH 9.2 borate buffer (23).

Mammalian Cell Culture

HeLa Cell Culture and Synchronization

HeLa cells were grown in suspension culture (at 3-6 X 10^5 cells/ml) in Joklik-modified Eagle's minimal essential medium supplemented with 7% calf serum and were synchronized by two successive treatments with 2 mM thymidine (162). The two, 14 hour, 2 mM thymidine treatments were spaced 9 hours apart. Rates of DNA synthesis were monitored by measuring the incorporation of [^{3}H]-thymidine into TCA-precipitable material in a 30 minute pulse (162). DNA synthesis was inhibited by treating the

cell culture with 1 mM hydroxyurea for 1 hour, beginning four hours after release from the second thymidine block. Cells (hydroxyurea treated or untreated) were harvested at various times after release from the second thymidine block.

HL60 Cell Culture and Differentiation

Human promyelocytic leukemia cells (HL-60 cells) were cultured in liquid suspension in RPMI 1640 medium supplemented with 10% fetal calf serum. HL60 cells were plated at a cell density of 2 X 10^5/ml, grown for 24 hours to a cell density of 3 X 10^5/ml and harvested by centrifugation. For differentiation of HL60 cells, cultures at a density of 3 X 10^5/ml were treated with TPA at a final concentration of 16 nM, and harvested after periods of 1, 2, 3, 4, and 5 days.

3T3L1 Cell Culture

Mouse 3T3L1 cells were maintained as subconfluent cultures in DMEM containing 5% calf serum in a moist, 5% CO2, 37°C incubator.

Transfection and Transient Expression of Recombinant DNA in 3T3L1 Cells

Transfection of 3T3L1 cells was carried out as described by Gorman et al. (56). Four hours prior to transfection, 3T3L1 cells in monolayer at 20-40% confluency were refed with fresh medium. The calcium phosphate/DNA

complex was prepared as described by Graham and Van der Eb (58) using 10 μg of plasmid DNA and 10 μg of salmon sperm DNA as carrier. Four hours after the addition of the calcium phosphate/DNA complex the medium was removed and the cells subjected to a one minute shock with 15-20% glycerol (v/v) in medium containing 10% fetal calf serum. Incubation was continued in fresh medium for 40-48 hours. Following incubation, the cells were harvested and total cellular RNA was isolated and analyzed by S1 nuclease protection analysis.

Selection of Stable Polyclonal 3T3L1 Cell Lines

3T3L1 cell monolayers were transfected as above using 20:1 molar quantities of construct and pSV2neo (158) without using carrier DNA (20 μg total DNA quantity). Selection of cells was carried out as described by Southern and Berg (158) using Geneticin (G418 sulfate). The medium plus drug was changed every three to four days until resistant colonies grew to approximately 1 cm in diameter. Polyclonal cell lines were established by combining resistant colonies as follows: the medium from each plate (containing from 2-20 resistant colonies) was removed, and the plates were treated with 2 ml trypsin (0.25% in buffered saline) for 2-5 minutes. The cells were rinsed from the surface of the plate and a 0.2 ml aliquot of the heterogeneous cell suspension was transferred to a plate containing fresh

medium and was maintained as a subconfluent culture in DMEM containing 5% calf serum in a moist, 5% CO_2, 37°C incubator. Ten polyclonal cell lines were established from the transfection of each of the constructs described above and are numbered P1 through P10. Cell lines 105-P7, 110-P10, and 109-P7 were chosen for further analysis (see chapter 5) based upon their ability to differentiate to adipocytes.

Induction and Differentiation of 3T3L1 Cells

Induction of 3T3L1 cells to adipocytes was carried out as described by Bernlohr et al. (7). 3T3L1 cells were grown to confluence and 3 days post confluence were given fresh DMEM containing 10% fetal calf serum, 0.22 μM insulin, 0.6 μM dexamethasone and 0.5 mM 3-isobutyl-1-methyl-xanthine. Forty-eight hours later the medium was replaced with fresh DMEM supplemented with 10% fetal calf serum. Adipocyte conversion was detected within 8 days of induction.

Cell Culture for Chromosome Localization

Isolation, propagation and characterization of parental human and murine cells as well as the human-mouse somatic cell hybrids was carried out as previously described (81,82,136) in Dr. Carlo Croce's laboratory at Temple University School of Medicine, Fels Research Institute, Philadelphia, PA. The human chromosomes (either partial or

complete chromosomes) retained in the rodent-human hybrids used in determining the chromosomal location of the human H2B histone gene are schematically illustrated in Figure 4-10B. I would like to acknowledge Shirwin Pockwinse, Dr. Kay Huebner, Dr. Linda A. Cannizzaro and Dr. Carlo Croce for their work on the chromosomal localization of the H2B-GL105 gene.

THP-1 Cell culture

Acute monocytic leukemia cells, THP-1 (173), were maintained in suspension cultures in RPMI medium 1640 supplemented with 5% fetal calf serum and kanamycin (60 μg/ml).

CHAPTER 3

ISOLATION AND CHARACTERIZATION OF A cDNA FROM A HUMAN HISTONE H2B GENE WHICH IS RECIPROCALLY EXPRESSED IN RELATION TO REPLICATION-DEPENDENT H2B HISTONE GENES DURING HL60 CELL DIFFERENTIATION

Introduction

Replication-dependent histone genes do not contain introns (164) and their mRNAs are structurally simple; they are not polyadenylated and have short 5' leader and 3' trailer sequences (77). However, replication-dependent histone mRNAs do have a characteristic 3' stem-loop motif (164). Replication-dependent histone genes are coordinately expressed during the cell cycle and their expression is coupled with DNA synthesis (3,74,126,130,131,176). The abundance of the replication-dependent human histone mRNAs is regulated at both the transcriptional and post-transcriptional levels (reviewed in 150,164).

In contrast, replacement histone genes may contain introns (20,182,183) and their mRNAs, which are polyadenylated and frequently contain long 5' leaders and 3' trailers, are structurally more complex than their replication-dependent counterparts (20,28,45,71,72,182,183). Although the processes governing replacement histone mRNA levels are not well understood, it is clear that their mRNAs

(10,19,25,45,71,89,152,155) as well as tissue-specific histone mRNAs (27,34,64,83,84,93) are not always regulated in a replication-dependent manner.

In these studies we cloned and characterized a cDNA from a variant human histone H2B gene which has a complex pattern of regulation with respect to the HeLa cell cycle and HL60 cell differentiation. Our results reveal a reciprocal relationship during the onset of HL60 differentiation between the expression of the HHC289 H2B gene and the replication-dependent H2B genes.

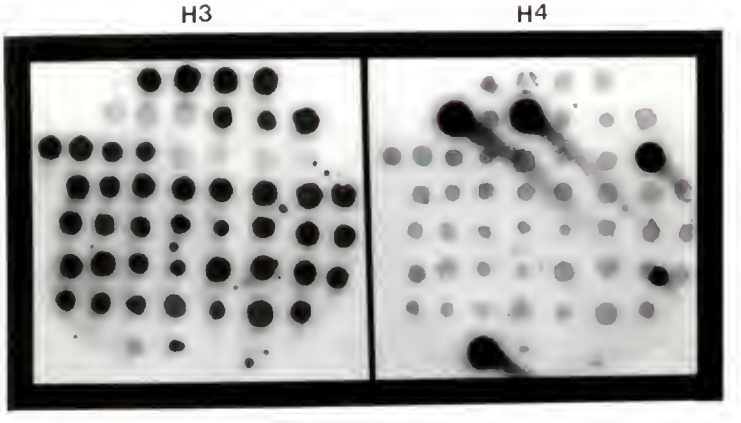
Results

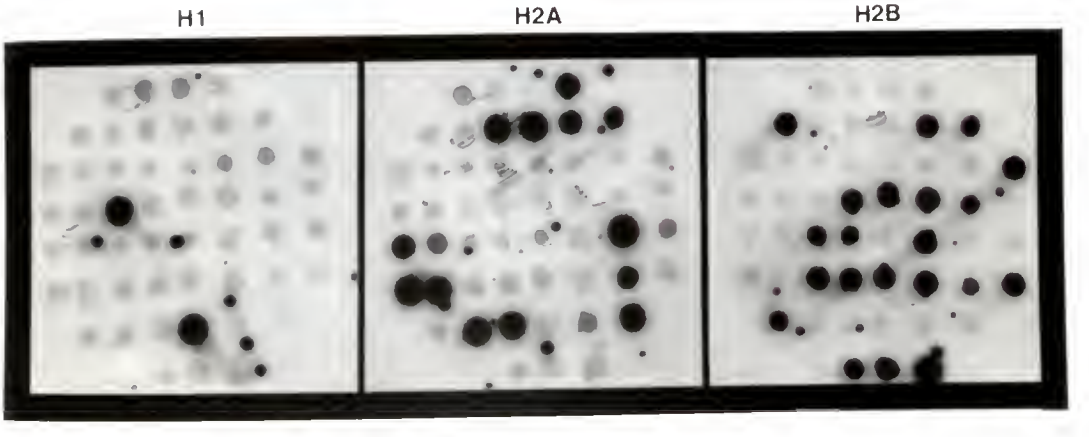
Isolation of an H2B cDNA Clone From a λgt11 Poly A+ cDNA Library

A λgt11 human liver cDNA library was screened with the ^{32}P-labeled human histone probes indicated in Figure 2-1. The library screening was carried out as indicated in Figure 2-2. Upon completion of the third round of screening 151 H3/H4 positives and 158 H2A/H2B/H1 positive plaques had been obtained. Using the human histone probes described in Figure 2-1, 51 H3/H4 positives and 51 H2A/H2B/H1 positive plaques were separated according to individual histone class by plaque dot analysis (Figure 3-1). Two H1 clones, 9 H2A clones, 22 H2B clones, 26 H3 clones and 5 H4 clones were identified. No clone gave a positive signal with more than one histone probe (Figure 3-1).

Figure 3-1. Plaque dot hybridization analysis of putative positive clones from the λgt11 poly A+ cDNA library screening

Upon completion of the third round of λgt11 library screening (see Figure 2-2) 51 H3/H4 positives and 51 H2A/H2B/H1 positive plaques were separated according to individual histone class by plaque dot analysis. Spot assay analysis was carried out using 0.5 μl aliquots of λ bacteriophage dilutions ~10^3 pfu. Upon plaque formation 2 filter lifts were prepared from the H3/H4 plate and 3 from the H2A/H2B/H1 plate and each hybridized to an individual histone ^{32}P-labeled probe (see Figure 2-1) as indicated above each panel. The filters were then washed and autoradiography performed using Kodak XAR-5 film.


H3
H4


H1
H2A
H2B

Restriction Endonuclease Mapping of Positive Clones Isolated From the λgt11 Poly A+ cDNA Library Screening

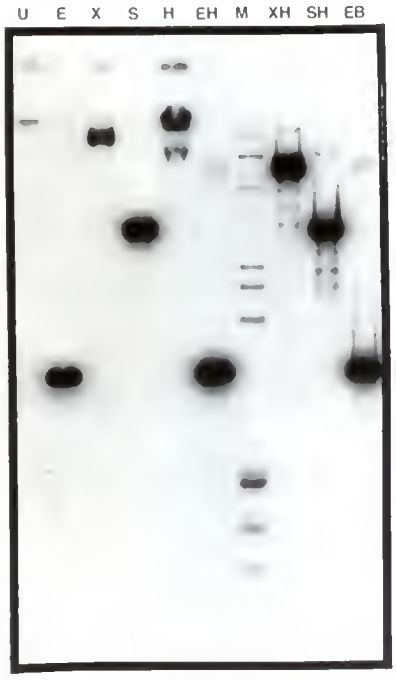
Twelve clones suspected of containing human histone genes were picked for further analysis (two from each histone class as well as two randomly picked additional clones) and mapped using a combination of restriction endonuclease double digestions and southern blot analysis (using the probes in Figure 2-1) as illustrated in Figure 3-2 for clone λHHC185. A restriction map of λHHC185 is illustrated at the top of Figure 3-2. Restriction endonuclease maps of the 12 positive clones isolated from the λgt11 poly A+ cDNA library and chosen for further analysis are illustrated in Figure 3-3. Insert sizes for the positive recombinant clones ranged from 490 nt for λHHC176 to 2160 nt for λHHC289.

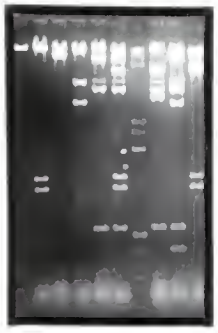
Sequencing Strategy for the Positive Clones Isolated From the λgt11 Poly A+ cDNA Library Screening

To evaluate whether any of the positive recombinant clones contained histone coding sequence, the largest clone in each of the histone classes was chosen for sequence analysis. The arrows in Figure 3-4 represent regions of the various λgt11 clones which were sequenced by Sanger dideoxy sequencing. Three of the clones (λHHC289, λHHC227 and λHHC4) were found to contain histone protein coding sequence

Figure 3-2. Restriction endonuclease Southern mapping of λHHC185

Each lane of a 0.8% agarose gel was loaded with 3 μg of λHHC185 DNA restricted with: E, Eco RI; X, Xho I; S, Sac I; H, Hind III; EH, Eco RI/Hind III; XH, Xho I/Hind III; SH, Sac I/Hind III; EB, Eco RI/Bam HI. Lane U was loaded with 3 μg uncut λHHC185 DNA and lane M with 3'-labeled λ DNA digested with Eco RI and Hind III and with 3'-labeled pBR322 DNA digested with Hinf I. Following separation of the DNA fragments electrophoretically, the DNA was transferred to Zeta-probe nylon membrane and hybridized to a ^{32}P-labeled H2B probe (see Figure 2-1). A photograph of the above gel stained with ethidium bromide is shown to the right of the figure for reference. A restriction map of λHHC185 is illustrated at the top of the figure. λgt11 arms are represented by closed boxes at the termini of the restriction map. Restriction enzyme abbreviations are as follows: E=Eco RI; X=Xho I; S=Sst I.





λHHC 185 MAPPING

Figure 3-3. Restriction endonuclease maps of positive clones isolated from the λgt11 poly A+ cDNA library screening

Restriction endonuclease maps of 12 positive clones isolated from the λgt11 poly A+ cDNA library screening are illustrated. Restriction endonuclease sites are as depicted in the top right corner of the figure. λgt11 arms are represented by closed boxes at the termini of the each restriction map. A - denotes a fragment giving a positive hybridization signal with a histone coding probe.

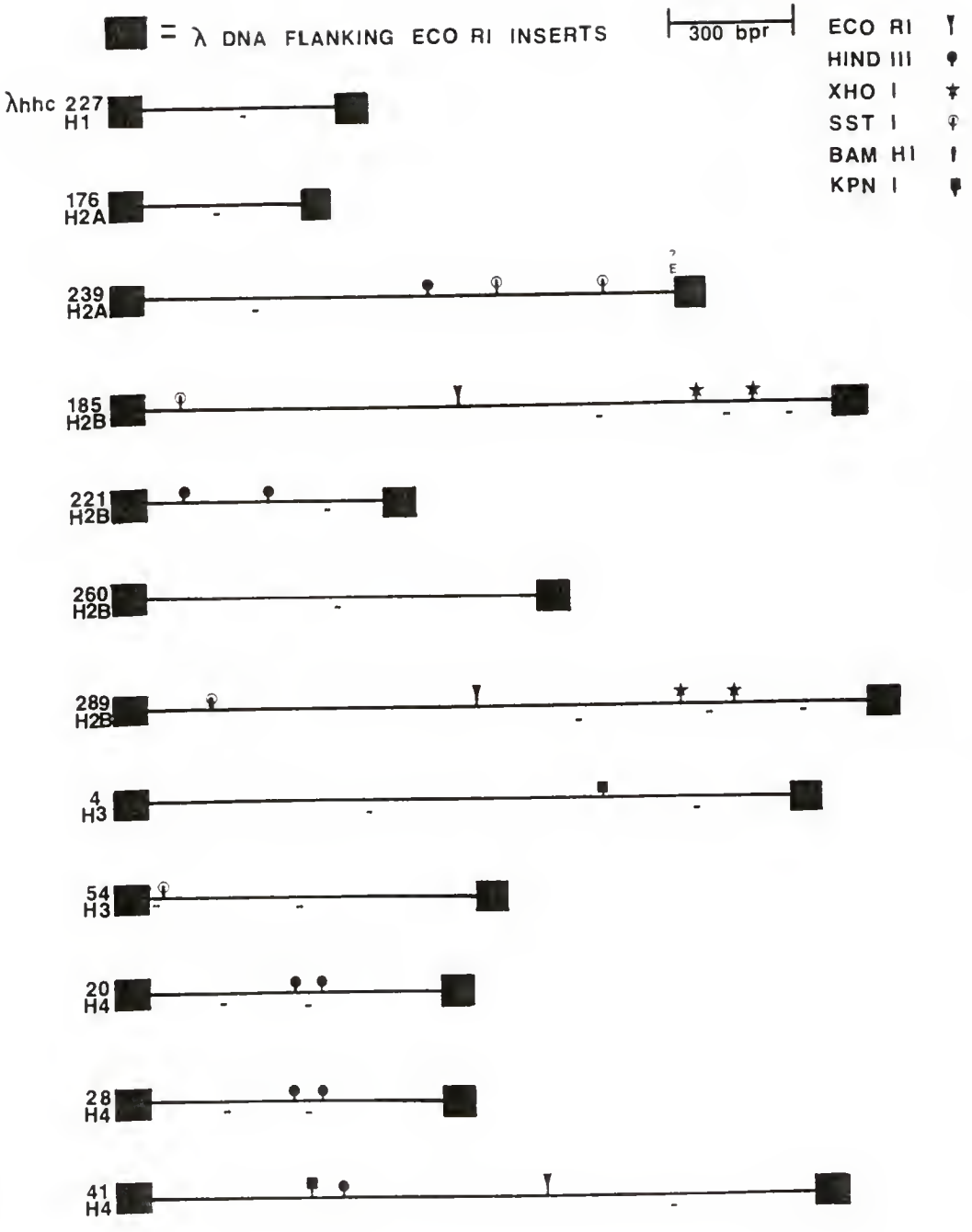
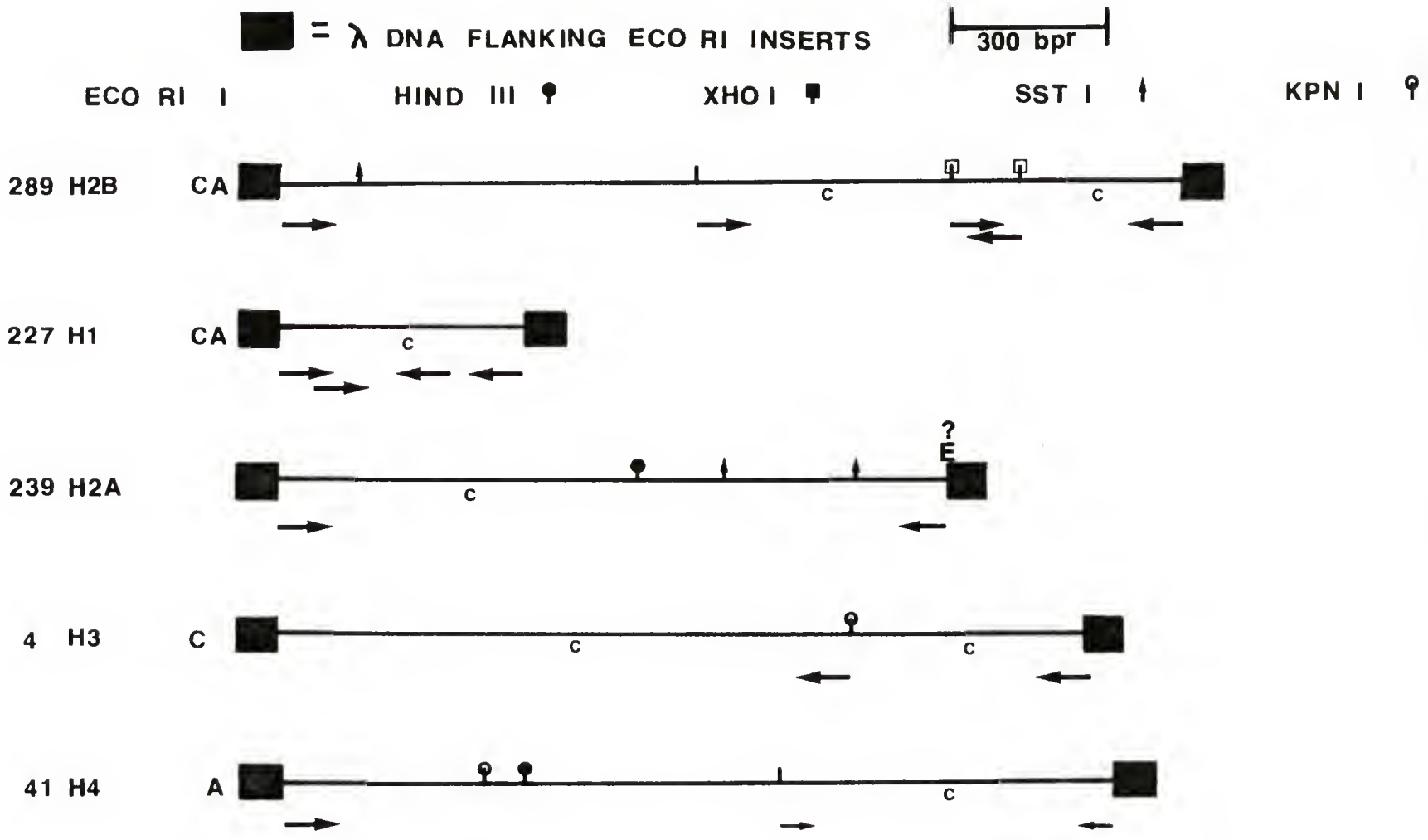

= λ DNA FLANKING ECO RI INSERTS
300 bpr
ECO RI
HIND III
XHO I
SST I
BAM HI
KPN I
λhhc 227
H1
176
H2A
239
H2A
185
H2B
221
H2B
260
H2B
289
H2B
4
H3
54
H3
20
H4
28
H4
41
H4

Figure 3-4. Sequencing strategy on the positive clones isolated from the λgt11 poly A+ cDNA library screening

Arrows represent regions of the λgt11 clones which were sequenced by Sanger dideoxy sequencing. A "c", below a map, indicates a restriction fragment which hybridized to a histone protein coding probe (see Figure 2-1) and a "C" to the left of a map marks the clones identified, by sequence analysis (146), to contain histone protein coding sequences. An "A" to the left of a map marks the clones identified, by sequence analysis, to contain poly(A) stretches at the end of the insert. Restriction endonuclease sites are as depicted at the top of the figure. λgt11 arms are represented by closed boxes at the termini of the each restriction map.


= λ DNA FLANKING ECO RI INSERTS
300 bpr
ECO RI I
HIND III
XHO I
SST I
KPN I
289 H2B
CA
C
C
227 H1
CA
C
239 H2A
?
E
C
4 H3
C
C
C
41 H4
A
C

and 3 clones (λHHC289, λHHC227 and λHHC41) to contain a stretch of poly(A) at the terminus of their inserts (Figure 3-4).

Sequencing of a Poly A^+ H2B cDNA

Of the 22 cDNA clones containing H2B histone sequences obtained from the initial screening of the lambda library four were mapped using a combination of restriction endonuclease double digestions and southern blot analysis and the clone with the largest insert (Figure 3-3, λHHC289 - 2119 nt) that also contained a poly(A) tract (Figure 3-4) was then selected for detailed sequence analysis. Regions of the human H2B histone cDNA λHHC289 which were sequenced are indicated by arrows in Figure 3-5 and the nucleotide sequence is presented in Figure 3-6(A). The λHHC289 cDNA encodes H2B amino acids 25-125 and the translation stop codon. Also contained in the cDNA sequence is the poly(A) addition sequence, AATAAA, located 1776 nt downstream from the translation stop codon, and a poly(A) stretch located 16 nt downstream of the poly(A) addition sequence. The protein coding region is GC rich (63%) whereas the 3' non-translated trailer is relatively GC poor (45%). Shown in Figure 3-6(B) are pertinent restriction sites and the λHHC289 fragments used as probes for northern blot analysis.

Figure 3-5. Sequencing strategy for the human H2B histone cDNA λHHC289

Arrows indicate regions of the human H2B histone cDNA λHHC289 which were sequenced by Sanger dideoxy sequence analysis (146). The H2B protein coding region is illustrated by a hatched box. Restriction enzyme abbreviations are as follows: E, Eco RI; X, Xho I; S, Sst I. λgt11 arms are not represented in this illustration.

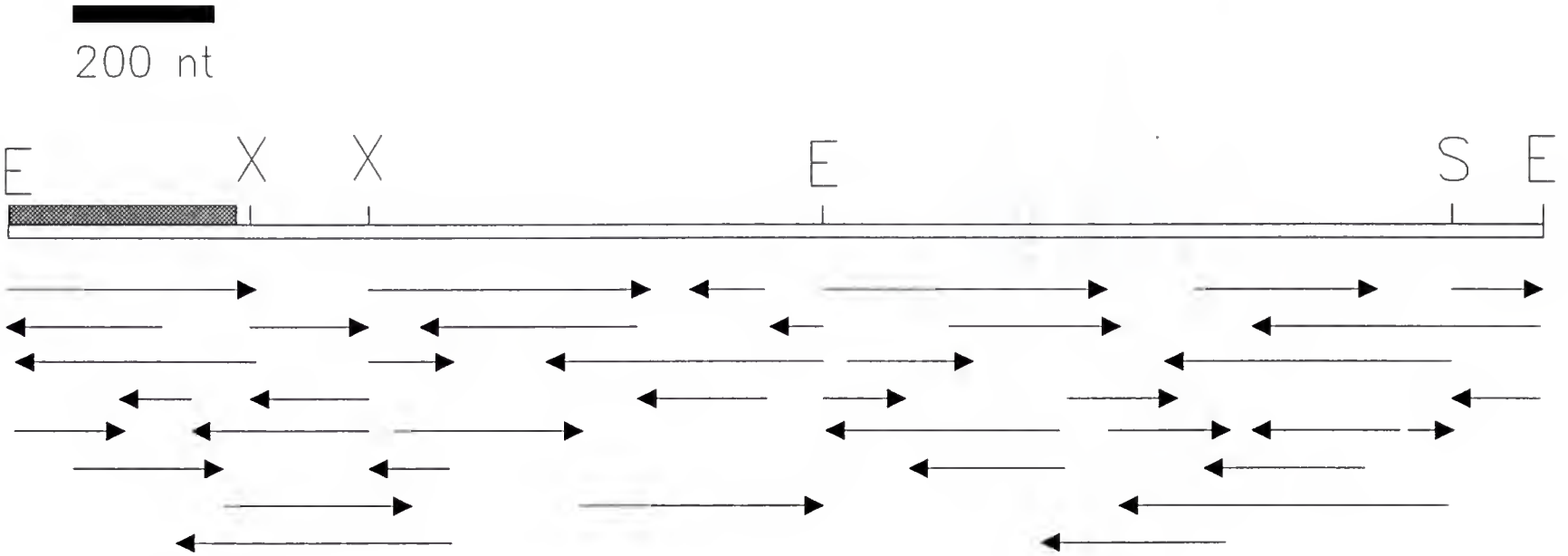

200 nt
E
X
X
E
S
E

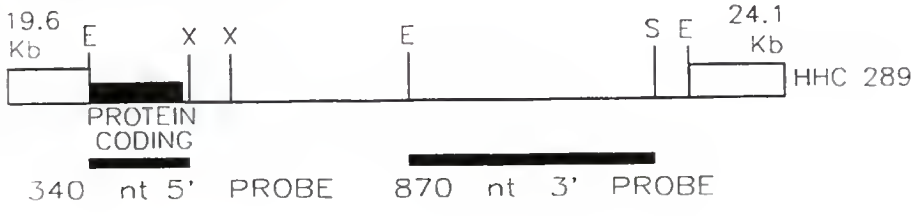
Figure 3-6. (A) Nucleotide and deduced amino acid sequence of the human H2B histone cDNA λHHC289. (B) Restriction map of the cDNA λHHC289

(A) The λHHC289 cDNA encodes H2B amino acids 25-125 and the translation stop codon. Also contained in the λHHC289 cDNA sequence is the poly A^+ addition sequence, **AATAAA**, located 1776 nt downstream from the protein stop codon and a poly A^+ stretch located 16 nt downstream from the last nucleotide of the poly A^+ addition sequence. Solid double underlined (___) sequences represent potential splice sites. (B) The H2B protein coding region is illustrated by a closed box and the λgt11 arms are represented by the open boxes. Solid bars below the restriction map illustrates λHHC289 probes used for northern analysis. Restriction enzyme abbreviations are as follows: E=Eco RI; X=Xho I; S=Sst I.

A

```
  GAC GGC AAG AAG CGC AAG CGC AGC CGC AAA GAG AGC TAC TCC ATC      45
25Asp Gly Lys Lys Arg Lys Arg Ser Arg Lys Glu Ser Tyr Ser Ile

  TAC GTG TAC AAG GTG CTG AAG CAG GTC CAC CCC GAC ACC GGC ATC      90
40Tyr Val Tyr Lys Val Leu Lys Gln Val His Pro Asp Thr Gly Ile

  TCG TCC AAG GCC ATG GGC ATC ATG AAC TCC TTC GTC AAC GAC ATC     135
55Ser Ser Lys Ala Met Gly Ile Met Asn Ser Phe Val Asn Asp Ile

  TTC GAG CGC ATC GCG GGA GAG GCT TCC CGC CTG GCG CAC TAC AAC     180
70Phe Glu Arg Ile Ala Gly Glu Ala Ser Arg Leu Ala His Tyr Asn

  AAG CGC TCC ACC ATC ACA TCC CGC GAG ATC CAG ACG GCC GTG CGC     225
85Lys Arg Ser Thr Ile Thr Ser Arg Glu Ile Gln Thr Ala Val Arg

  CTG CTG CTG CCC GGC GAG CTG GCC AAG CAC GCC GTG TCC GAG GGC     270
100Leu Leu Leu Pro Gly Glu Leu Ala Lys His Ala Val Ser Glu Gly

  ACC AAG GCG GTC ACC AAG TAC ACC AGC TCC AAG TGA GTC CCT GCC     315
115Thr Lys Ala Val Thr Lys Tyr Thr Ser Ser Lys ***

GGACCTGGCG CTCGCTCGCT CGAGTCGCCG GCTGCTTGAC TCCAAAGGCT CTTTTCAGAG  375
CCACCCACCT AATCACTAGA AAAGAGCTTG TTCACTTATT CCCTTAGTTT CTTTTCATAA  435
AGTAAGTTAT CTTAGTGTGA AGGTCATGGG AAATGGCATA CGTAGCTTTT TAACTATTTG  495
GAACTCGAGG TCCCCAGTGC GTCATTGGAT TTGCTTTTGA ATCTAGAGCG TGTCTTTACT  555
CATTGTGCTG CTTAGCCTTC CCAGGAGTCG GTTCTCAATT AGGCTGTTGG GAATCCGCCT  615
CTTTACCCGC CCCCACTCCC GCCCCACACG CGCCCTGGTG GCTCCTTGGG TCTGTTTCAT  675
TCTAAAACGA AGTGGCTGAG TTCGGCTGTC ATTTAAGAGA ACTCCAGGAC ACAATTCAGC  735
CCGGGTTCCG CAAACACTGC GTGACAGCTC TGTATGACTG ACGCTTGGCA GCAGCTTTTG  795
TGTCCGGTCA CCAGTTCTGC CGTGCGATGG GGCCTCCTGT GGATACCAGC CGTTCTGTGT  855
ATTTTGGACG AAGGCGGCAG CCGGGTCCCA GCCTTGTCCT GATTGGGCGA CAAGAATATT  915
CAAAATTCTG CGCCTTTTTC TAATTTGTAG ATTTCAGTTT CCGTCGTTCA CTTTGAGACT  975
TTGAAATTCC TATTTCTCAT TTTGTTGATA ATTTCTGCAT TTAATGGTCT GTGCTTTAAA 1035
TGGTAACGCT ACGGCCCCAG GTCACTGCGA GGCACTTACC ATGTAGATAC GGGCTCAAAA 1095
GTCACCTCTC AGAGACCTAC GTCATCCACT CAGGAATTCG CGCCTCTCAT ACTTGCCTGT 1155
CTCATTTTAT CTTCCTTCTA GCAGCTGTCT GAAATTGGTT CGTCTGTTTT CTTGTTTATG 1215
GTATTCTCAA GCCCTTGACA GACCGGCTAG TGTGGTTTTC CCGTGCATCT TCAGCCTGGC 1275
ACATTATGGA CACTTAAATA CTACGTATTG ATCTAATATT GTTGGGTTAA TTTTTCCATC 1335
CCACCCTTTT CTTAATCGCT TCCGTGGATG GATGAAGGGT GCTGTTCATT TCCATTAGAT 1395
GTATGTGAAG GCACAGTGAA AATGGAAATG TTCTTGGAGC TACTTCCTCA AAATGTATCC 1455
TTAGTCACCT CAGTGCAACA GCTGGGAGGG GGCCGTGTTA AGATTTTTTT TGCTACAAAG 1515
AGGAGGTGGC AATGGTAGAT CCACCCTTAT GCTTCTCAGT TTAGCATAAC CTCTTATGGA 1575
TTTTCATCAA ATTCAGCGTG TTGGTCACTG GAAAGAGCCT TTTCCTTCTC CTTTTCTTAC 1635
TCTCCCCTCA TGGTGTTCCC CTCTTAAAGG AGAGGAGCTT TTAATTTACA CTTACCACCT 1695
CATTTGCTTT TCTGGAGGCC ATGCAATATA GGCGGGACTA CAGAGTTAAT CTCCTTTTTA 1755
CAAATGAGGC CAAGAGAAGC CTCATTGGTT CACAGTCATG CAGCTCATAC TGTCCACCCT 1815
TGTATTCTCA GATGCAGGAC AATTGCATTT TAGTTTTATT TTGTGGAGGT GCAGAATATT 1875
TACTCTTTCT GTCCAACCCT TGATTCTGCC GAGGAAGACA CTGATGGTTT GATGAGTGAT 1935
TCAGCTGTTT TTGGCTAAGG GCTTTTGGAG CTGATGGCAG GGGTTTGATG AATCCAAATG 1995
AGCTCTAGAC ATTATCACAG ACTGAATAGA TCTTAACTGT CTCCTACATG TGTGTTTTCA 2055
AATGTGTATA GATGCTATTG TTATTAATAA AGTTACCAAT TAATTTAAAA AAAAAAAAAA 2115
AAAA                                                              2119
```

B



Comparison of the λHHC289 Predicted Amino Acid Sequence With Other H2B Amino Acid Sequences

In Figure 3-7(A) is shown a comparison of selected amino acids from the predicted histone H2B sequence of λHHC289 with a multi-species consensus H2B protein sequence (184), a human genomic sequence (134) and several chicken genomic sequences (27,59,70). The amino acids shown vary at the indicated positions among the genes reported in Figure 3-7(A). These amino acids are either species-specific, that is, they are conserved among known chicken H2B genes but may differ between the chicken and other species reported in Figure 3-7(A) (amino acids 18, 21, 25 and 26), or subtype-specific which may vary among H2B protein variants within a species or between species (amino acids 30, 31, 32, 39, 60, 94, 122 and 124). The amino acid sequence data for human H2B histones are too incomplete to permit classification of the HHC289 H2B protein as a replication-dependent, replacement, or tissue-specific variant. However, the predicted protein is 97% similar to the replication-dependent HHG39 H2B protein (134) with amino acid differences at positions 39, 94 and 124 (Figure 3-7(A)) and identical to the multi-species consensus H2B sequence compiled by Wells (184). The λHHC289 predicted amino acid sequence is also similar to the pHh4A/pHh4C H2B sequence reported by Zhong et al. (194), except in the region of amino acids 27 through 33.

Figure 3-7. (A) Comparison of the λHHC289 histone H2B amino acid sequence with the sequences of other H2Bs. (B) DNA sequence comparison of the 3' untranslated region of selected histone sequences

(A) **A**, λHHC289 human cDNA; **B**, multi-species consensus H2B protein sequence (184); **C**, λHHG39 human genomic sequence (134); **D**, pBBA-3.0 chicken genomic sequence (59); **E**, pPP2d-4.0 chicken genomic sequence (59); **F**, pKR1a-1.3, pPP2d-2.3, pRR3c-3.5, pRR2e-3.5 and pBRA-5.4 chicken genomic sequences (59); **G**, λCH-02 and λCH-01/λCH-05 chicken genomic sequences (70); **H**, pStH2B-16 chicken genomic sequence (27). Dashed lines indicate undetermined amino acids due to incomplete nucleotide sequence information and an asterisk indicates an amino acid which is identical to the λHHC289 predicted amino acid in the same position. The amino acid position is indicated by the bold number at the top of each column and the origin of each sequence is indicated in the right hand column. Amino acids which do not vary among the genes reported above are not illustrated. (B) **1**, λHHC289 human H2B cDNA; **2**, pKR1a-1.3 chicken H2B genomic sequence (59); **3**, pBBA-3.0 chicken H2B genomic sequence (59); **4**, λHHG39 human H2B genomic sequence (134); **5**, pHh4A/pHh4C human H2B genomic sequence (194); **6**, pHh5G human H2A genomic sequence (194); **7**, pST519 human H3 genomic sequence (111); **8**, human H3.3 genomic sequence (182); **9**, human H2A.Z cDNA sequence (72); **0**, pStH2B-16 chicken genomic sequence (27). Numbers flanked by dots indicate the number of bases contained between the two adjacent reported sequences. A blank space is a gap created to facilitate alignment. A plus sign symbolizes the presence of a poly A^+ tail in the mRNA and a question mark indicates the lack of data concerning polyadenylation or the number of bases contained between two adjacent reported sequences. Arrows indicate the conserved 3' stem-loop motif sequence. The conserved sequences are displayed in the top line, in bold print.

A

	18	21	25	26	30	31	32	39	60	94	122	124	
A.	---	---	Asp	Gly	Lys	Arg	Ser	Ile	Gly	Ile	Thr	Ser	Human
B.	Leu	Ala	*	*	*	*	*	*	*	*	*	*	Multi
C.	Val	Ala	*	*	*	*	*	Val	*	Val	*	Lys	Human
D.	Val	Thr	Gly	Asp	*	*	Ala	*	Ser	*	*	*	Chicken
E.	---	---	---	---	---	---	---	*	*	*	Ile	*	Chicken
F.	Val	Thr	Gly	Asp	Arg	Lys	*	*	*	*	*	*	Chicken
G.	Val	Thr	Gly	Asp	*	Lys	*	*	*	*	*	*	Chicken
H.	Val	Thr	Gly	Asp	Arg	*	Thr	*	*	*	*	*	Chicken

B

TGCA CCCAAA GGCTCTTTTCAGAGCCACC GAGCT AATAAA

1. TGA..28..TGCA..17..TCCAAA GGCTCTTTTCAGAGCCACC..18..GAGCT..1800..AATAAA..15..+
2. TAG...5..TGCG..15..CCCAAA GGCTCTTTTCGGAGCCACC..16..GGGCT..????.............?
3. TAA...5..TTCT...9..CCCAAACGGCTCTTTTCGGAGCCCAA..13..GGGCT..????.............?
4. TTA...9..TGCA..14..CCCAAA GGCTCTTTTCAGAGCCACT..16..GAGCTATATACTGACATGTGAAGAT
5. TAA..13..TGCA...9..CCCTAACGGCTCTTTTAAGAGCCACC..16..GAGCTGGTGCTTGTATTTCCTCCTC
6. TGA..15..TGCA..36..CAGCAA GGCTCTTTTCAGAGCCACC..17..GAGCTGTTGTGCTTTGGATTATGCC
7. TAA...1..TGTA..26..CCCAAA GGCTCTTTTCAGAGCCACC..18..TAGCTGTGATAATTTTTTGTTGTCT
8. TAA..23..TTCA.. TTCTCAAAAAAAAAAAAAAAATTTCTCTTCTTCCTGTTAT...422..ATTAAA..15..+
9. TAA...8..TGGA.. TTCCTTGTTATCTCAGGACTCTAAATACTCTAACAGCTGT...302..AATAAA..16..+
0. TAG...5..TGCG..15..CCCAAA GGCTCTTTTCAGAGCCACC..16..GAGTG..????.............+

Comparison of 3' Non-translated Sequences From λHHC289 and Other Histone cDNAs and Genes

The 3' non-translated sequences from λHHC289 and other histone cDNAs and genes are illustrated in Figure 3-7(B). The histone 3' stem-loop element (77) is present in all of the poly A^- histone sequences (59,111,134,194). Included in this comparison are three poly A^+ human histone sequences (72,182) and one poly A^+ chicken tH2B sequence (27); of these, only λHHC289 and the chicken tH2B sequence contain a 3' stem-loop motif. The λHHC289 H2B and the H2A.Z sequences (72) contain the AATAAA poly(A) addition element (49,135) while the H3.3 sequence (182) contains an ATTAAA element, also implicated in polyadenylation (68,90) (see Figure 3-7(B)). However, the tH2B sequence does not appear to contain either polyadenylation element (27). In addition to containing a 3' stem-loop motif, λHHC289 is distinct from other known human poly A^+ histone sequences as it contains the longest 3' trailer--1798 nt as compared to 510 for H3.3 (182) and 376 for H2A.Z (72)

HHC289 mRNA is Expressed Throughout the HeLa Cell Cycle and During Inhibition of DNA Synthesis

To determine the extent of cell cycle regulation of the HHC289 H2B histone gene, we first measured the level of HHC289 mRNA throughout the HeLa cell cycle. Presented in Figures 3-8 and 3-9 are the results of northern blot analysis of poly A^+ RNA isolated from synchronized HeLa

Figure 3-8. HHC289 mRNA levels during the HeLa cell cycle

Poly A^+ RNA was isolated from synchronized HeLa cells, prior to (0) and after (1, 2, 3, 4, 6, 8 and 10 hours) release from a double thymidine block, as well as from unsynchronized HeLa cells in logarithmic (L1) growth. In a separate experiment poly A^+ RNA was isolated from unsynchronized HeLa cells, in logarithmic growth, which had been treated with the DNA synthesis inhibitor hydroxyurea (HU) as well as from cells which were not treated (L2). 2.0 μg of each RNA sample was size fractionated, passively transferred to Zeta-Probe nylon membrane, prewashed, prehybridized and hybridized to the following ^{32}P-labeled probes: 870 nt Eco RI/Sst I 3' fragment from λHHC289 (panel A); plasmid RGAPDH-13 (52) containing GAPDH sequences from rat (panel B). The filters were then washed and autoradiography performed using Kodak XAR-5 film. Lane M contains RNA markers purchased from Bethesda Research Labs. The sizes of the various bands are indicated, in Kb, by the numbers on the right side of the figure.

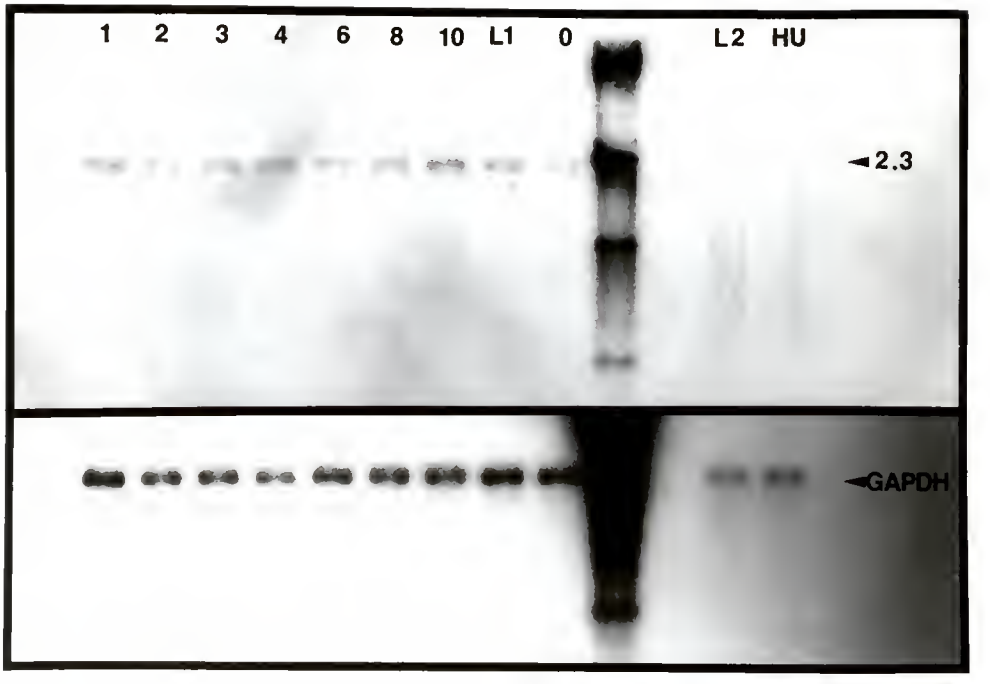
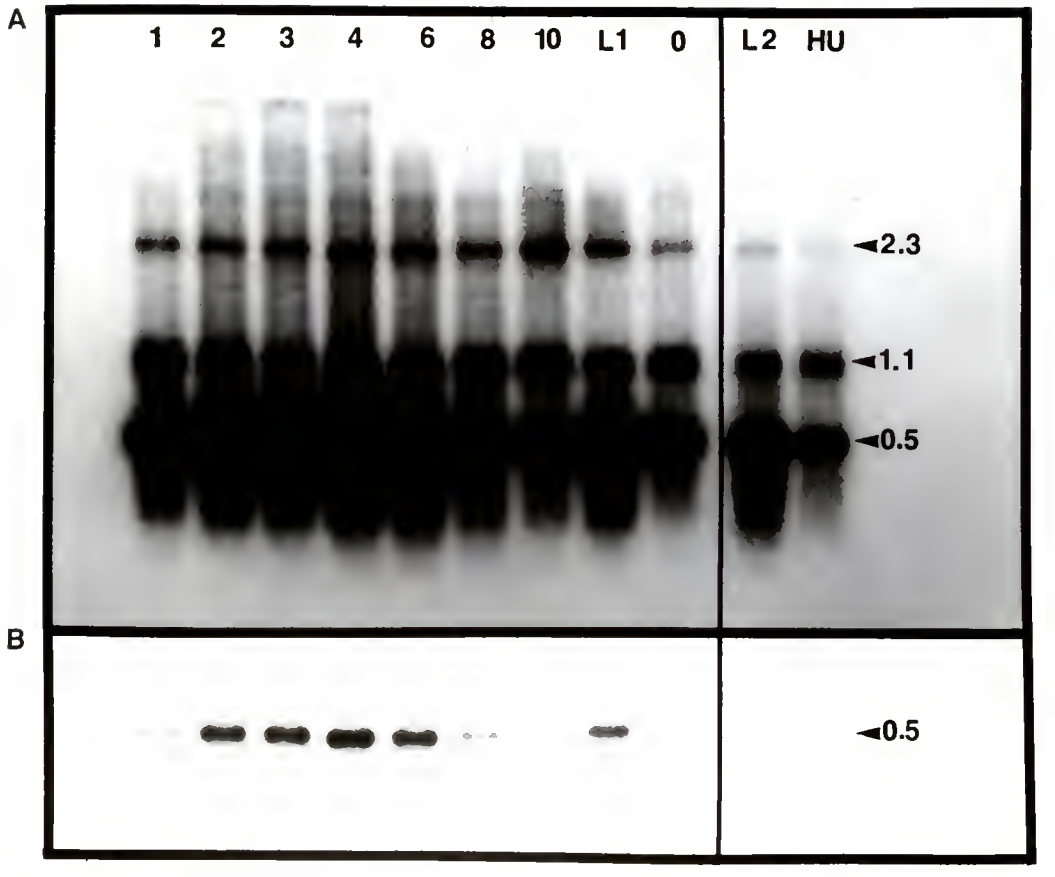

1
2
3
4
6
8
10
L1
0
L2
HU
2.3
GAPDH

Figure 3-9. H2B mRNA levels during the HeLa cell cycle

Northern blot analysis was carried out on the same HeLa samples used in Figure 3 with the ^{32}P-labeled 340 nt Eco RI/Xho I fragment from λHHC289 as probe (panel A). Panel B contains a shorter exposure of the 0.5 Kb bands from panel A. The sizes of the various bands are indicated, in Kb, on the right side of the figure.


A
1
2
3
4
6
8
10
L1
0
L2
HU
2.3
1.1
0.5
B
0.5

cells, prior to and after release from a double thymidine block, as well as from unsynchronized HeLa cells in logarithmic growth and after treatment with the DNA synthesis inhibitor hydroxyurea. The peak of S phase, as determined by ^{3}H-thymidine incorporation, occurred at 4-5 hours after release from the second thymidine block (data not shown).

To follow the mRNA levels of all H2B species, two different probes from λHHC289 were used. One probe was the 340 nt Eco RI/Xho I fragment (Figure 3-6(B)) which spans the H2B protein coding region and has strong sequence similarity with other reported H2B sequences. The second probe was the 870 nt Eco RI/Sst I fragment (Figure 3-6(B)) that spans the distal half of the 3' non-translated trailer. Northern blot analysis using the 3' specific probe revealed a 2300 nt band that was present throughout the HeLa cell cycle (Figure 3-8(A)). As an internal control for the amount of mRNA per lane, this blot was reprobed with the ^{32}P-labeled plasmid RGAPDH-13 (52), which contains rat cDNA sequences coding for GAPDH (Figure 3-8(B)). GAPDH mRNA levels have been shown in our lab to remain constant throughout the HeLa cell cycle. In cells in which DNA synthesis was inhibited with hydroxyurea, the level of the 2300 nt HHC289 mRNA species decreased by approximately 50 percent (Figure 3-8(A)), as indicated by densitometry and normalization to GAPDH mRNA levels (Figure 3-8(B)).

When northern blot analysis was carried out using the protein-coding fragment from λHHC289 (Figures 3-9(A) and 3-9(B)), H2B mRNAs were observed at 2300 nt and 1100 nt, in addition to low molecular weight H2B species migrating at approximately 500 nt. It should be noted that a single round of selection with oligo dT-cellulose greatly enriches for poly A^+ RNA but does not totally remove all the poly A^- RNA. The 500 nt H2B mRNAs were detected throughout the HeLa cell cycle but their levels clearly fluctuated as a function of the cell cycle (Figures 3-9(A) and 3-9(B)). Levels of the 1100 nt and 2300 nt mRNAs remained relatively constant throughout the cell cycle, in comparison to the 500 nt replication-dependent mRNAs (Figure 3-9(A)). Densitometric analysis revealed that after treatment of proliferating HeLa cells with hydroxyurea the levels of the HHC289 mRNA (2300 nt), as well as the 1100 nt mRNA, dropped to approximately 50 percent of their noninhibited levels, whereas the 500 nt replication-dependent mRNAs decreased by 95% (Figure 3-9(A)). An additional H2B clone with a different restriction pattern from that of λHHC289 has been isolated from a poly A+ cDNA library (see clone λHHC221 in Figure 3-3) which suggest that the 1100 nt replication independent H2B mRNA species is not the transcription product of HHC289 gene but possibly that of the putative HHC221 gene.

Transcription of the HHC289 Histone Gene During the HeLa Cell Cycle

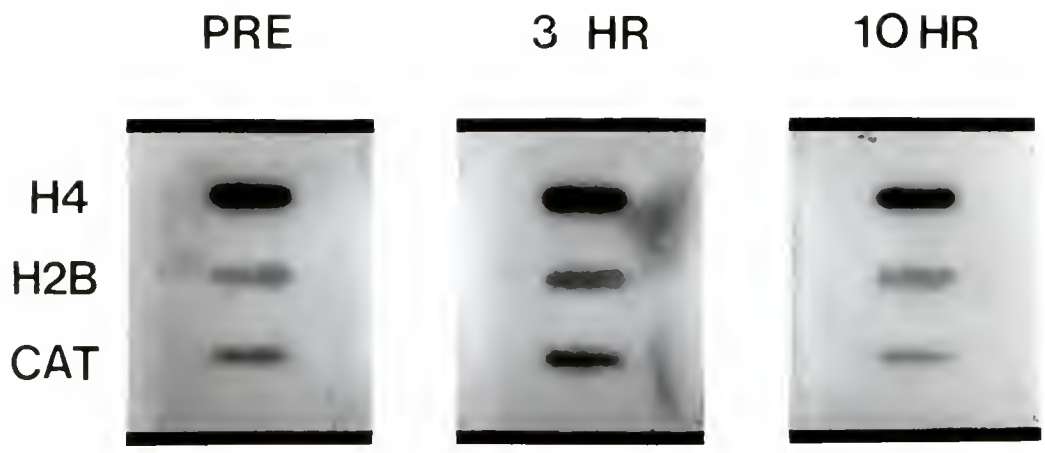
To determine the extent to which transcription contributes to the cell cycle regulation of HHC289 gene expression, we carried out *in vitro* run-on transcription analysis of nuclei isolated from synchronized HeLa cells after release from a double thymidine block (Figure 3-10). During S phase (3 hours) transcription from the HHC289 gene was two fold higher than its level outside of S phase (10 hours). In comparison, we observed that during S phase total H4 histone and H4/CAT fusion gene transcription increased 2 to 3 fold over the levels outside of S phase (Figure 3-10).

HHC289 mRNA is Expressed in a Reciprocal Relationship With Replication-Dependent H2B mRNAs During the Onset of HL60 Cell Differentiation

We examined the level of HHC289 mRNA following induction of HL60 promyelocytic leukemia cells along the monocytic pathway, as proliferation ceases and monocyte-specific gene expression is initiated. RNA was isolated from proliferating HL60 cells and from cells at various times after induction of differentiation with TPA. Northern blot analyses, using the 3' specific probe (Figure 3-11(A)) and the protein-coding fragment (Figure 3-12), revealed that the 1100 nt and 2300 nt H2B histone mRNAs, present in very low levels in the proliferating HL60 sample, were induced upon TPA treatment. Based on densitometric analysis the

Figure 3-10. Transcription of the HHC289 histone gene during the HeLa cell cycle

The polyclonal HeLa cell line 201p1, which contains an H4 histone-CAT fusion gene, was synchronized via double thymidine block. Cells were harvested at the G1/S border (PRE), in mid S-phase (3 hours), and in G2/M (10 hours). Radiolabeled transcripts (10^7 dpm) from *in vitro* nuclear run-on transcription were hybridized to slot blots containing linearized plasmid DNAs: H4, replication-dependent human H4 histone gene (131); H2B, 1020 nt 3' *Eco* RI fragment from the λHHC289 replication-independent H2B cDNA; and CAT, H4 promoter/CAT fusion gene. The hybridized filters were then washed and autoradiography performed using XAR-5 or Cronex film and Cronex Lightning Plus screens at -70°C. Densitometric analysis was performed and the resultant values for transcription of the histone genes and H4/CAT fusion gene were normalized to those of the 18S ribosomal RNA gene (not shown). The results obtained in six similar experiments are represented in the table.



RATIO OF TRANSCRIPTION AT THREE HOURS RELATIVE TO TEN HOURS, t3/t10

ABSOLUTE	2.0 ± 0.94	n=6
NORMALIZED	2.1 ± 0.53	n=5

Figure 3-11. HHC289 mRNA levels during proliferation and differentiation of HL60 cells

Poly A^+ RNA, 1.0 μg, from proliferating (P) and differentiated (Diff.) HL60 cells was used for Northern blot analysis. RNA was isolated from differentiated HL60 cells at 1 (1d), 2 (2d) and 3 days (3d) after treatment with TPA. The ^{32}P-labeled probes were the 870 nt Eco RI/Sst I 3' noncoding fragment from λHHC289 (panel A) and the 550 nt Pst I insert from pGβ2m (38) (panel B).

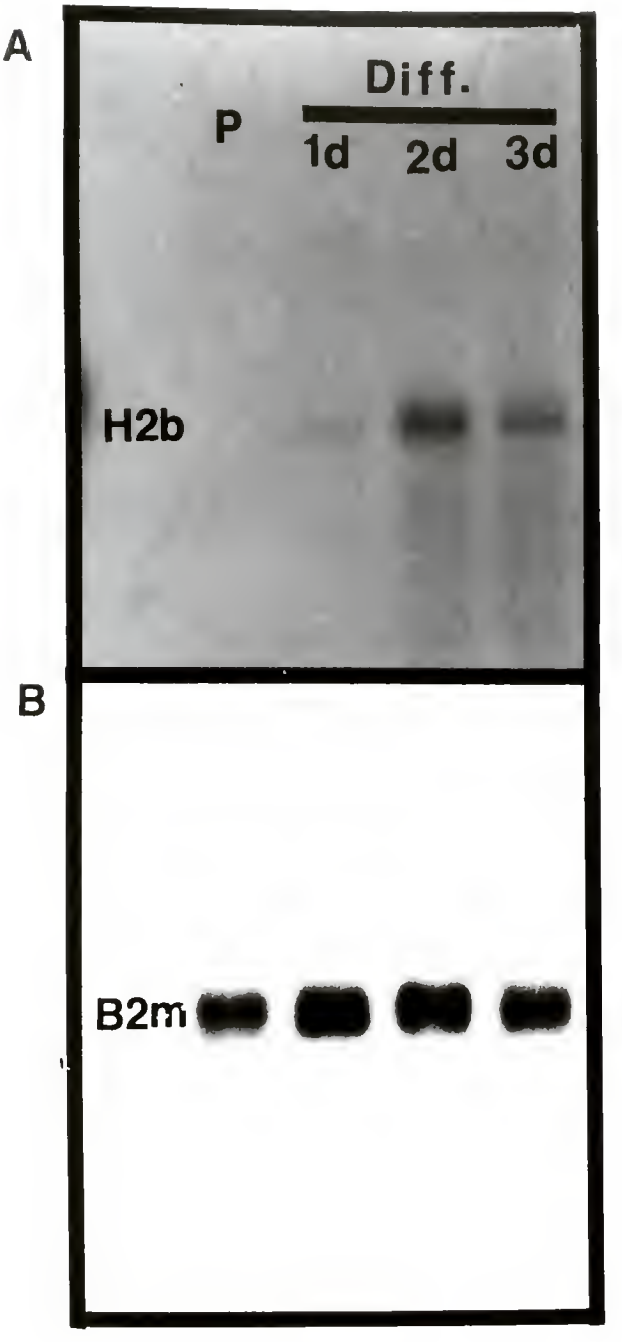
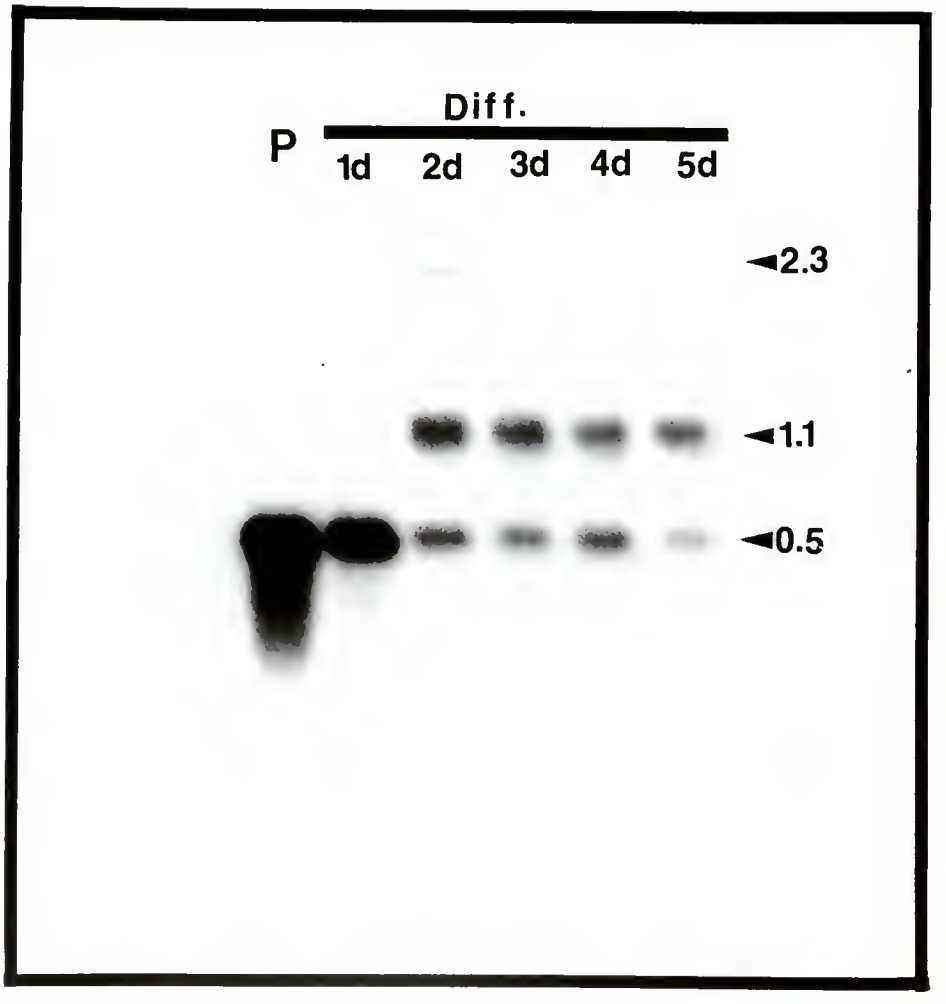

A
P
Diff.
1d
2d
3d
H2b
B
B2m

Figure 3-12. H2B mRNA levels during proliferation and differentiation of HL60 cells

Poly A^+ RNA, 1.0 μg, from proliferating (P) and differentiated (Diff.) HL60 cells was used for Northern blot analysis with the ^{32}P-labeled Eco RI/Xho I H2B coding-region fragment of λHHC289 as probe. RNA was isolated from differentiated HL60 cells at 1 (1d), 2 (2d), 3 (3d), 4 (4d) and 5 days (5d) after treatment with TPA. The sizes of the various bands are indicated, in Kb, on the right side of the figure.


Diff.
P
1d
2d
3d
4d
5d
2.3
1.1
0.5

2300 nt HHC289 mRNA was present at a 10-fold higher level by day 2 than in the proliferating sample (Figure 3-13). The 1100 nt mRNA, present at a very low level in the proliferating HL60 cells, also increased until day 2 (Figure 3-12). The 500 nt H2B mRNAs were detected at a high level in the proliferating HL60 sample but rapidly declined until at day 5 they had decreased approximately 100-fold from their level in the proliferating sample. As illustrated in Figure 3-13, a reciprocal relationship exists during HL60 differentiation between the high molecular weight H2B mRNAs, which increase 10-fold, and the 500 nt H2B mRNAs which decrease to less than 1% of their level in proliferating HL60 cells.

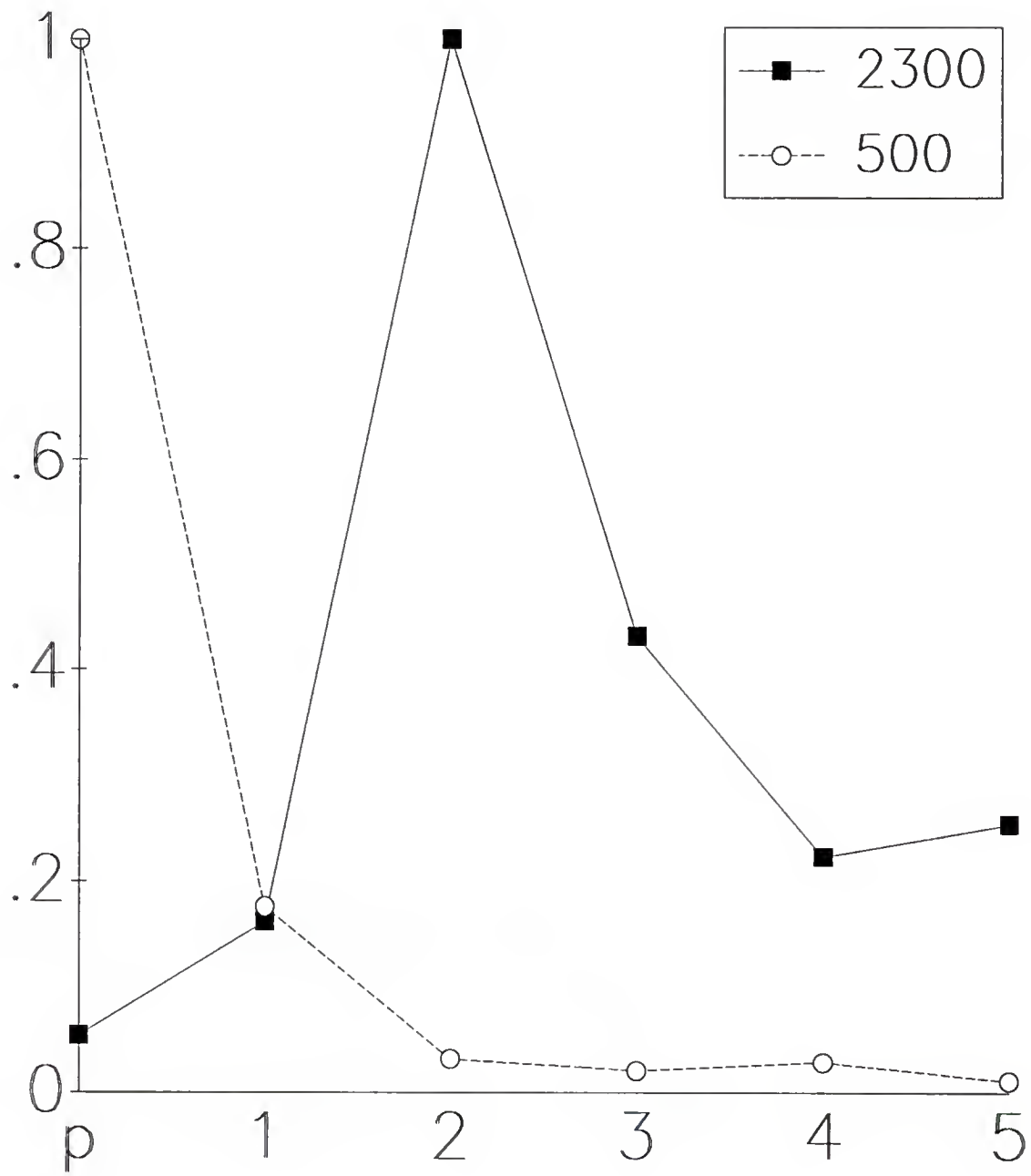
Discussion

Structural Analysis of the λHHC289 H2B cDNA

Although comparison of the nucleotide sequence of λHHC289 with several other H2B histone sequences revealed that the protein coding region is highly conserved, the striking differences in the 3' trailing sequences suggest that the λHHC289 cDNA is unique among H2B histone sequences described thus far. The λHHC289 H2B cDNA is the only H2B human histone sequence reported which contains both the highly conserved histone 3' stem-loop structure (77) and a poly(A) addition sequence (49,135) followed by a poly(A) stretch, although Mannironi *et al.* (110) have reported an

Figure 3-13. Densitometric analysis of the 500 nt replication-dependent H2B mRNA and the 2300 nt HHC289 mRNA levels during differentiation of HL60 cells

Densitometric analysis of the 500 nt replication-dependent H2B mRNA (O,dashed line) and the 2300 nt HHC289 mRNA (■, solid line) levels during differentiation of HL60 cells. The mRNA levels are presented as a fraction of the maximum for each mRNA (vertical axis).


1
.8
.6
.4
.2
0
p
1
2
3
4
5
2300
500

H2A histone isoprotein (H2A.X) which is encoded by a poly A^+ mRNA containing the 3' stem-loop structure and a poly(A) addition sequence. λHHC289 is also distinct from the other poly A^+ histone sequences reported in that it contains the longest 3' trailer. The structural elements described for λHHC289 (the highly conserved histone 3' stem-loop structure and a poly(A) addition sequence followed by a poly(A) stretch) may play a role in conferring both replication-dependent and replication-independent properties.

Cell Cycle Regulation of the HHC289 H2B Histone Gene

When H2B mRNA levels were measured throughout the HeLa cell cycle, the 2300 nt and 1100 nt mRNAs did not undergo cell-cycle fluctuations as did the replication-dependent histone mRNAs (3,72,130,131). In addition, the level of the 2300 nt HHC289 mRNA species in proliferating HeLa cells dropped only 50% after treatment with hydroxyurea, suggesting incomplete coupling to DNA synthesis. This is consistent with results on the rat testis-specific H4 and sea urchin α-histone H4 mRNAs containing the 3' stem-loop motif, which have been observed to be stable in the presence of hydroxyurea (64,89). The steady state level of the poly A+ 2300 nt mRNA is much lower than the steady state level of cell cycle dependent H2B mRNAs in proliferating cells (less than 5%) (Figure 3-9 and data not shown), however, quantitative comparisons are difficult due to the fact that

several H2B genes contribute to the cell cycle dependent H2B mRNA pool. In vitro run-on transcription analysis of nuclei isolated from HeLa cells revealed that transcription from the HHC289 gene is two fold higher during S phase than outside of S phase, whereas during S phase total H4 histone and H4/CAT fusion gene transcription increased 2 to 3 fold over the levels outside of S phase (Figure 3-10). These results suggest that transcriptional regulation of HHC289 expression is similar to that of replication-dependent histone genes.

Our HeLa cell cycle data raise the question: Does the internal 3' stem-loop motif influence HHC289 mRNA processing and/or stability or are these controlled primarily by other 3' trailer elements, such as the large size of the 3' trailer sequence as well as its poly(A) tail? It is also possible that nucleotide sequences in protein coding regions are involved in histone mRNA degradation (89). The level of the 2300 nt HHC289 mRNA species is very low in proliferating HeLa and HL60 cells; this observation raises the possibility of a dual processing pathway in which the majority of the HHC289 transcripts are cleaved to the internal 3' stem-loop motif, leaving only a minority in the 2300 nt form. This is consistent with reports of histone genes with alternative pathways of mRNA 3' end processing that produce independently regulated mRNAs from a single gene (27,29-31). The possibility of a dual processing pathway for the HHC289

mRNA is further supported by the presence of the sequence AAAGTAAGT (position 434-442 in Figure 3-6(A)), located 51 nt 3' to the stem-loop motif, which is nearly identical to the 5' splice site consensus (18). The λHHC289 sequence also contains a potential 3' splice site (18), TGTGTATAGAT (position 2057-2068), that if spliced to the AAAGTAAGT 5' splice site would result in a poly A^+ mRNA similar in size to the bulk of H2B mRNA observed in cycling cells. This potential splice junction conforms to the "GT-AG" rule (18) and is immediately preceded by a pyrimidine-rich region and a branchpoint sequence (63), TCTTAAC, 33 nt upstream. Stauber et al. (159) observed possible processing of an H4 mRNA at a similar 5' splice site consensus located 41 nt 3' to the stem-loop motif to an undefined 3' splice site further downstream. The 1100 nt H2B mRNA observed when northern blot analysis was carried out using the protein-coding fragment from λHHC289 (Figure 3-9(A)) could result from expression from another H2B gene or may represent dual processing of transcripts from the HHC289 gene.

Regulation of HHC289 mRNA During Differentiation in HL60 cells

Northern blot analysis of poly A^+ RNA isolated from proliferating and differentiated HL60 cells demonstrated that the HHC289 mRNA is regulated in a very different manner than the mRNAs for most other H2B histones. The 2300 nt HHC289 mRNA and replication-dependent H2B histone mRNA

levels appear to be regulated in a reciprocal fashion when HL60 cells are terminally differentiated. A portion of the 500 nt replication-dependent H2B histone mRNA detected may arise from the HHC289 gene due to utilization of the 3' stem-loop structure or by a splicing event in the 3' trailer (see chapter 4). These results confirm and extend our previous studies demonstrating the downregulation of replication-dependent histones and their reciprocal relationship with a replication-independent H2B gene during the onset of HL60 cell differentiation (37,152,161). The increase in 2300 nt HHC289 mRNA levels during differentiation may provide H2B histone proteins required for the reorganization and condensation of chromatin taking place during differentiation.

A striking similarity exists between the upregulation of the 2300 nt HHC289 H2B mRNA during differentiation in HL60 cells and the upregulation of the chicken tH2B mRNA in pachytene spermatocytes (27). Both the HHC289 mRNA and the tH2B mRNA contain the 3' stem-loop motif in addition to a poly(A) tail (27). Replication-dependent H2B mRNAs are also transcribed from the same gene as the tH2B mRNA but are processed by an alternate pathway of mRNA 3' end formation (27). The existence of alternate 3' processing pathways for this chicken H2B gene raises the possibility that a similar dual processing may be operative for the HHC289 gene, which would be consistent with the presence of potential splice

sites in the λHHC289 sequence (Figure 3-6). However, the presence of the 3' stem-loop motif suggests that the HHC289 gene may utilize the U7 mediated histone processing pathway (see chapter 4) in addition to the poly (A) addition pathway.

We speculate that the HHC289 H2B histone gene encodes H2B proteins which are used in three situations: i) during DNA synthesis when deposition of new histone proteins is required for packaging DNA into chromatin; ii) for replacement of old histones on active genes or of damaged histones in the genome; iii) during differentiation when the cell's chromatin is being reorganized or condensed and when deposition of new histone proteins may be required. We suggest that the primary role for variant histone genes such as HHC289 is to provide histones for replacement and reorganization or condensation when the bulk of replication-dependent histone mRNAs have been degraded and the majority of histone protein synthesis has been shut down. Although the HHC289 mRNA has various structural features that make it unique among human H2B histone mRNAs, such as a large 3' flanking region and a polyadenylation sequence, it also shares elements with replication-dependent histone mRNAs, such as the 3' stem-loop motif. These elements may provide the means for alternative mRNA processing as the metabolic requirements of the cell are altered. However, it is also possible that the dual processing pathways are constitutive

and that the mRNAs are differentially or selectively degraded.

CHAPTER 4

ALTERNATIVE 3' END PROCESSING IS INVOLVED IN MODULATING EXPRESSION OF A HUMAN HISTONE H2B.1 VARIANT GENE, LOCATED ON CHROMOSOME 1, IN RESPONSE TO CHANGES IN THE PROLIFERATIVE STATE OF THE CELL

Introduction

The expression of replication-dependent histone genes is coupled with DNA synthesis and the abundance of these mRNAs is regulated at both the transcriptional and post-transcriptional levels (3,74,130,131,176 and reviewed in 113,151,163). Transcriptional regulation involves a 2 to 5 fold increase in the rate of transcription at the G1/S phase boundary, with a return to basal level by mid to late S phase (4,74 and reviewed in 113,151,163). Post-transcriptional regulation of these histone genes involves a rapid and selective destabilization of histone mRNAs toward the end of S phase or in response to inhibition of DNA synthesis (3,76,117,142). In contrast, the mRNA levels of replication-independent histone genes are not regulated in the same cell cycle dependent manner as their replication-dependent counterparts (10,19,25,45,71,89,152).

Although several replication-dependent human histone H2B genes and one pseudogene have been described (74,112,131,134,154,194), no replication-independent human

H2B genes have been reported. However, we have isolated and characterized a human H2B histone cDNA which is reciprocally expressed in relation to replication-dependent H2B histone genes during HL60 cell differentiation (35) (see chapter 3). Here we describe the cloning and characterization of this variant human H2B histone gene (GL105). This gene expresses alternative mRNAs that are regulated differentially during the HeLa cell cycle. The structural and organizational components of this H2B histone gene include consensus elements for DNA synthesis dependent histone genes as well as elements typical of constitutively expressed histone genes. The complex expression pattern of this H2B histone gene may be of functional importance in providing H2B.1 histone proteins in response to modifications of chromatin architecture that accompany alterations in gene expression.

Results

Isolation of an H2B Genomic Clone From a λEMBL4 Human Adult Lymphocyte DNA Library

To investigate the relationship between gene structure and the regulation of variant H2B mRNA levels, we used a variant H2B cDNA clone, λHHC289 (chapter 3), to probe a λ human genomic library. One complete equivalent of a λEMBL4 human genomic DNA library (8 X 10^5 phage) was screened with a ^{32}P-labeled H2B 3' non-translated probe (870 nt Eco RI/Sac I fragment isolated from λHHC289 (see Figure 3-6)). Upon completion of the third round of

screening 8 positive plaques had been obtained. Figure 4-1 illustrates a representative section of a film obtained during the third round of library screening.

Restriction Endonuclease Mapping of Positive Clones Isolated From the λEMBL4 Genomic DNA Library Screening

Four of the 8 positive clones suspected of containing human H2B histone genes were randomly picked for further analysis and mapped using a combination of restriction endonuclease double digestions, southern blot analysis and indirect end-labeled mapping as illustrated in Figure 4-2 for clone λHHG17E. Restriction endonuclease maps of the 4 putative genomic H2B clones, isolated from the λEMBL4 genomic DNA library screening and chosen for further analysis, are illustrated in Figure 4-3. Insert sizes for the positive recombinant clones ranged from 15 Kb for λHHG20E to 26 Kb for λHHG17E. A positive clone (λHHG5E) corresponding to the λHHC289 cDNA was selected for further analysis. The arrow in the upper right corner of Figure 4-1 points out clone λHHG5E during the third round of the genomic library screening. The 4100 nt Eco RI fragment cross-hybridized with the ^{32}P-labeled 340 nt Eco RI/Xho I protein coding fragment from λHHC289, and the 6100 nt Eco RI fragment cross-hybridized with the ^{32}P-labeled H2B 3' non-translated probe (870 nt Eco RI/Sac I fragment isolated from λHHC289 (see Figure 3-6)). The 4100 nt and 6100 nt Eco RI fragments from λHHG5E (Figure 4-3) were isolated and

Figure 4-1. Third round screening of a λEMBL4 human adult lymphocyte DNA library with a λHHC289 3' non-translated trailer probe

One complete equivalent of a λEMBL4 human genomic DNA library (8 X 10^5 phage) was grown on 14 150 mm diameter petri dishes and screened with a ^{32}P-labeled H2B 3' non-translated probe (870 nt Eco RI/Sac I fragment isolated from λHHC289 (see Figure 3-6)). Illustrated is a representative section of a film obtained during the third round of library screening. The arrow in the upper right corner of the figure indicates clone λHHG5E which was one of the 4 clones chosen for further analysis.


F117A
HHC289 H2B GENOMIC SCREENING / 900nt E-S
5E

Figure 4-2. Indirect end-labeled mapping of the putative genomic H2B clone λHHG17E

λ phage DNA (λHHG17E) was partially restricted with: E, Eco RI; B, Bam HI; X, Xho I; S, Sac I; or A, Asp I. Oligonucleotides complementary to the cos sequence of λ phage were 5' end labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP and hybridized to 0.5-1.0 μg of the partially restricted λ phage DNA. Following the hybridization the samples were loaded onto a 0.5% agarose TPE gel and electrophoresed for 24-28 hours at 40 watts with buffer recirculation. Following electrophoresis the gel was dried under vacuum for 1 hour at 80°C. Autoradiography was performed using pre-flashed Kodak XAR-5 X-ray film and a Cronex Lightning Plus screen at -70°C. Lane M1 was loaded with 3'-labeled λ Eco RI marker DNA and lane M2 with 3'-labeled λ Sal I marker DNA. The sizes of the marker fragments are indicated on the left side of the figure in Kb.

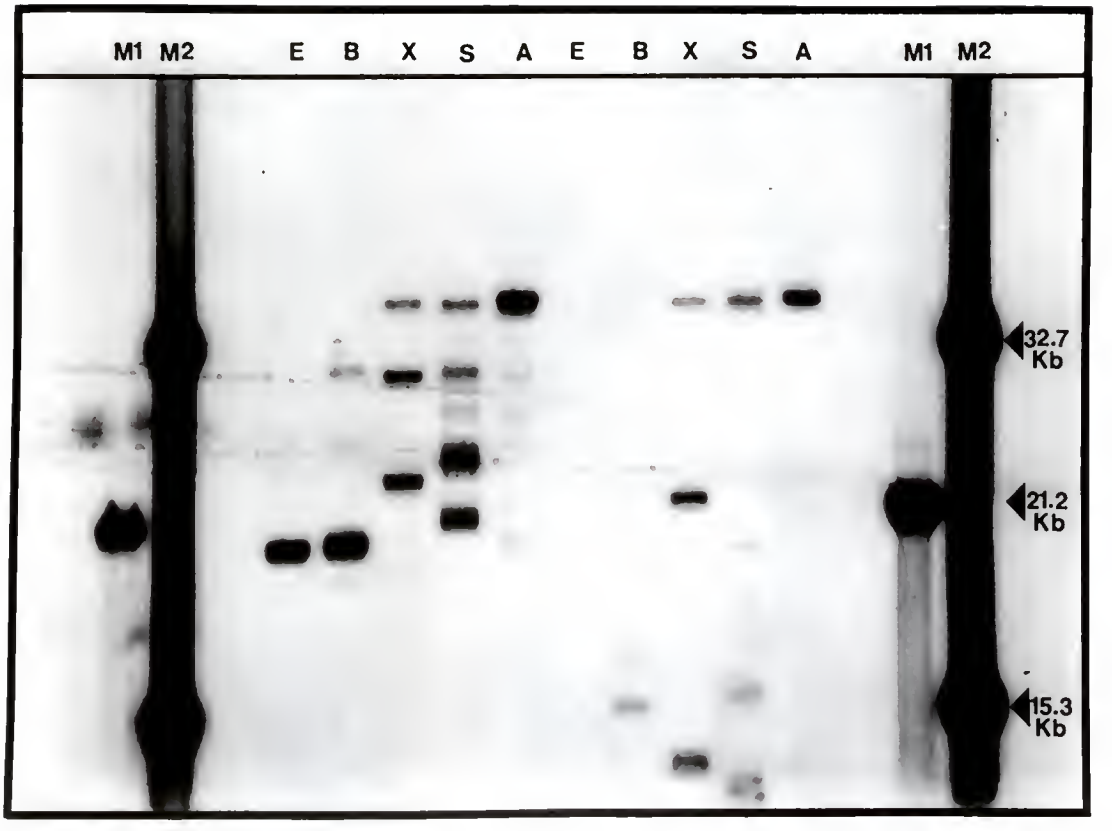
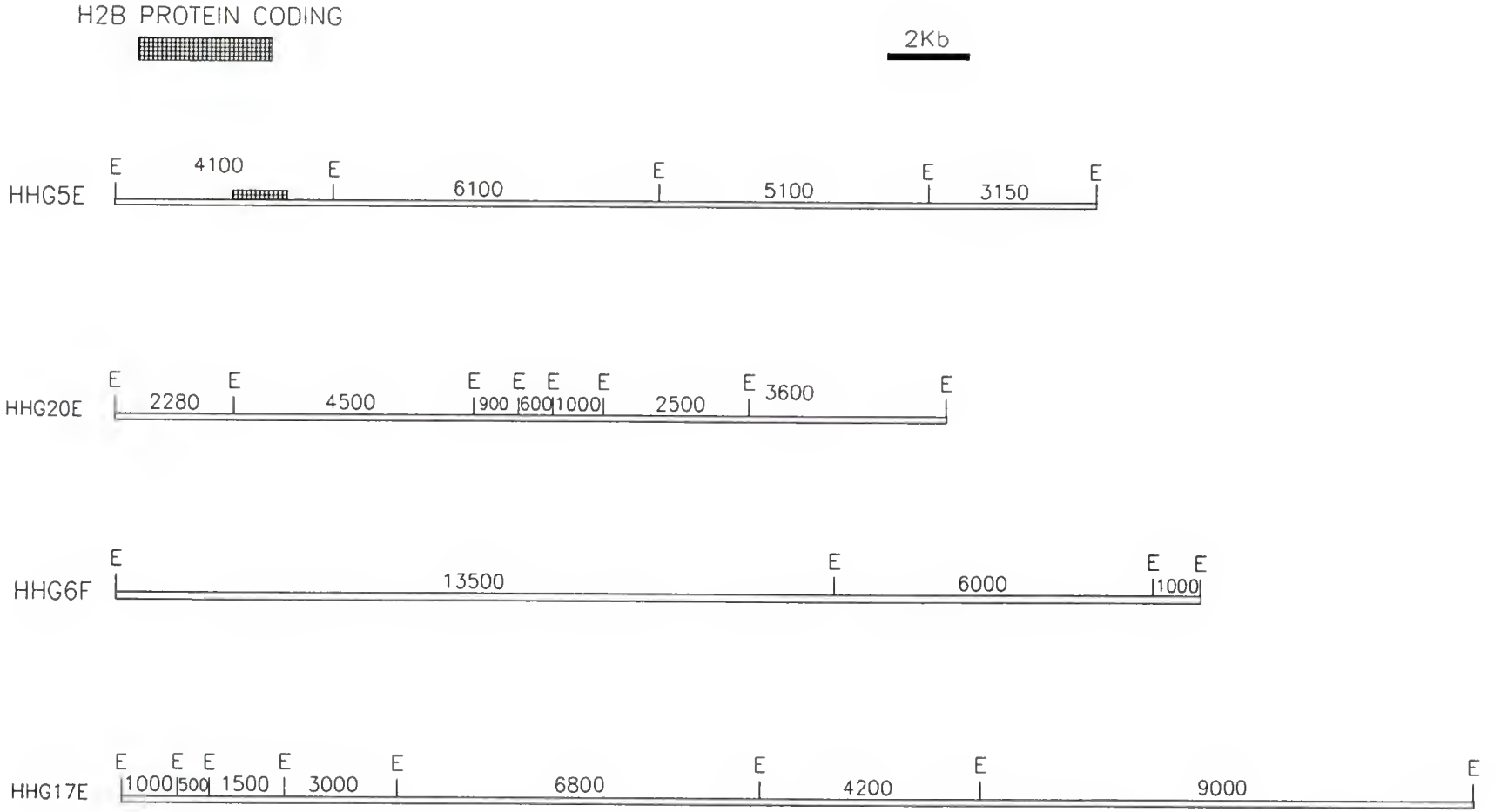

M1
M2
E
B
X
S
A
E
B
X
S
A
M1
M2
32.7 Kb
21.2 Kb
15.3 Kb

Figure 4-3. Restriction map of putative genomic H2B clones

Restriction endonuclease maps of 4 positive clones isolated from the λEMBL4 genomic DNA library screening are illustrated. The Eco RI restriction endonuclease sites (E) and the size of each Eco RI fragment are shown above each map. The H2B protein coding region of clone λHHG5E, which was chosen for sequence analysis, is illustrated by a cross-hatched box. The λEMBL4 arms are not represented in these maps.


H2B PROTEIN CODING
2Kb
HHG5E
E
4100
E
6100
E
5100
E
3150
E
HHG20E
E
2280
E
4500
E
900
E
600
E
1000
E
2500
E
3600
E
HHG6F
E
13500
E
6000
E
1000
E
HHG17E
E
1000
E
500
E
1500
E
3000
E
6800
E
4200
E
9000
E

cloned into pUC19 and the resulting subclones termed pGL101 and pGL102, respectively. Subclones pGL101R and pGL102R were also constructed and contain the same fragments as pGL101 and pGL102 but inserted into pUC19 in the opposite orientation. Additional restriction endonuclease mapping and southern blot analysis was carried out using these subclones as illustrated in Figure 4-4 for pGL201R.

Cloning and Nucleotide Sequence of the H2B-GL105 Gene

The sequence of a 2964 nt portion of λHHG5E was determined by dideoxy sequence analysis and revealed the presence of an H2A gene upstream of the H2B gene (Figure 4-5). The 6100 nt Pst I fragment, illustrated in Figure 4-5 and containing the entire H2B gene from λHHG5E, was cloned into pUC19 and is termed pGL105. The nucleotide and deduced amino acid sequences of the human histone H2B-GL105 and a portion of the adjacent H2A histone GL101 are presented in Figure 4-6. The λHHG5E H2B gene encodes a protein identical to the somatic H2B.1 mouse protein in key amino acid positions (Ser^{14}, Ala^{21}, Asp^{25}, Lys^{27}, Gly^{60}, Asp^{68}, Gly^{75}, Glu^{76}), as reviewed by Zweidler (195). A restriction map of the genomic clone λHHG5E demonstrating the location of the H2B protein coding region is shown in Figure 4-7. The λHHG5E H2A/H2B gene pair share an intergenic region with a spacing of 329 nt between their protein coding regions and 231 nt between their cap sites (Figure 4-6). The H2A and

Figure 4-4. Southern blot analysis of the genomic H2B subclone pGL201R

Each lane of a 0.8% agarose gel was loaded with 500 ng of pGL201R DNA restricted with: E, Eco RI; ES, Eco RI/Sac I; EP, Eco RI/Pst I; EA, Eco RI/Asp 718; A, Asp 718; AS, Asp 718/Sac I; P, Pst I; S, Sac I. Lanes M were loaded with marker DNAs which were used to size the ethidium bromide stained DNA fragments (data not shown). Following separation of the DNA fragments electrophoretically, the DNA was transferred to Zeta-probe nylon membrane and hybridized to a ^{32}P-labeled probe (870 nt Eco RI/Sac I fragment isolated from λHHC289 (see Figure 3-6)).

pGL 201R

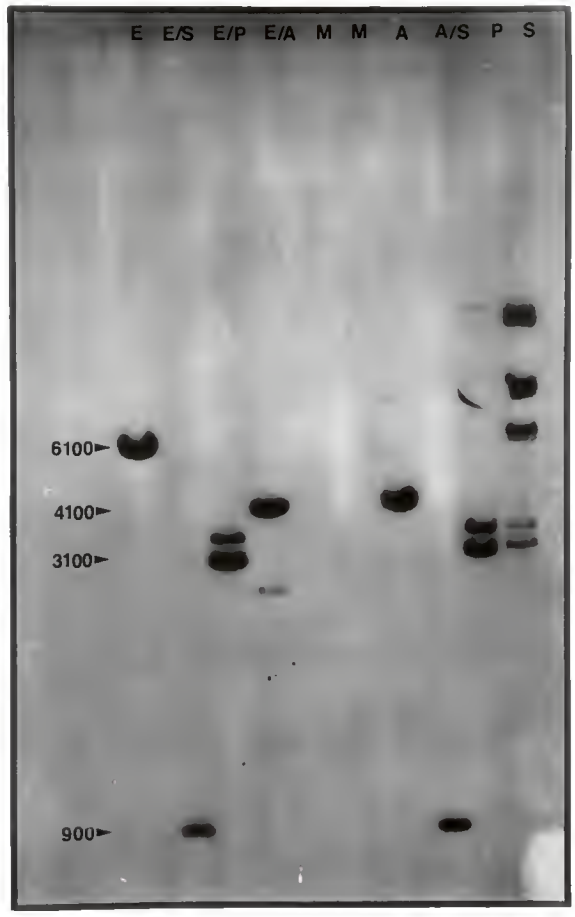


Figure 4-5. Sequencing strategy for the human histone gene H2B-GL105

Lines having filled arrowheads indicate regions of the human H2B histone genomic clone λHHG5E which were sequenced by Sanger dideoxy sequence analysis (146) and lines containing hollow arrowheads indicate regions sequenced using the EXOMETH DNA sequencing kit purchased from Stratagene. The dotted line with two filled arrowheads represent the region of λHHC289 which had previously been sequenced in both directions by Sanger dideoxy sequence analysis (146) (see Figure 3-5). The H2B protein coding region is illustrated by a hatched box. Restriction enzyme abbreviations are as follows: E, Eco RI; X, Xho I; S, Sst I; P, Pst I; S', Sph I; H, Hind III. λEMBL4 arms are not represented in this illustration.

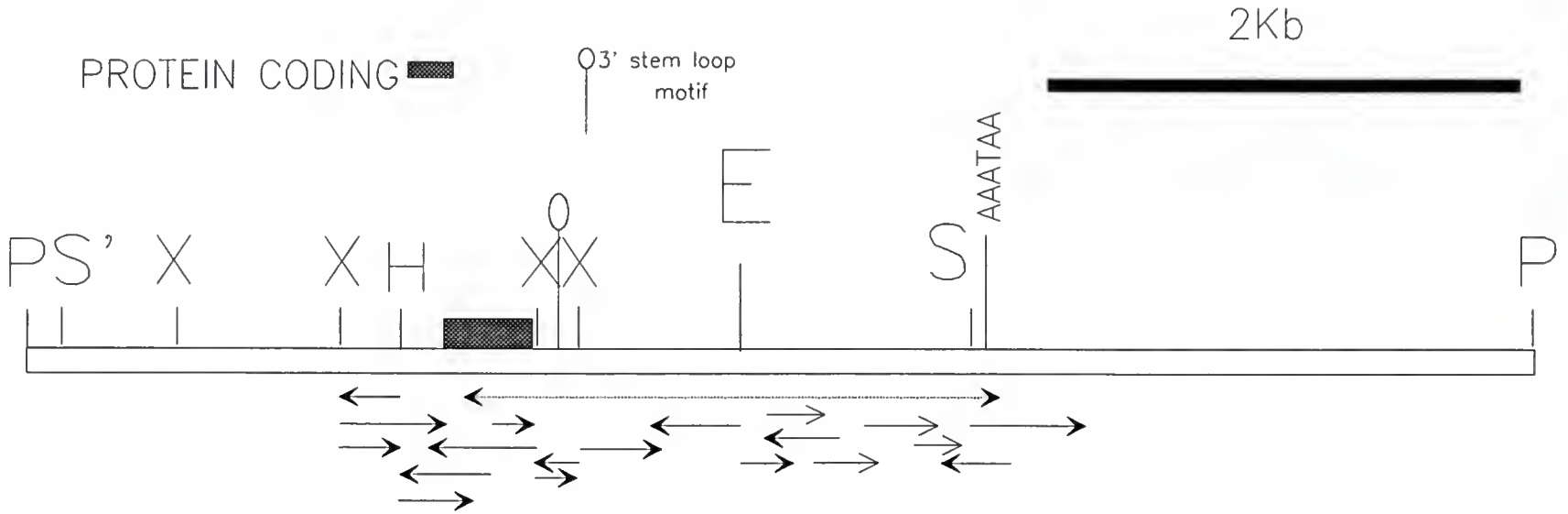

PROTEIN CODING
3' stem loop
motif
2Kb
AAATAA
PS' X X H X X E S P

Figure 4-6. Nucleotide and deduced amino acid sequences of the human H2B and H2A histone genes from the genomic clone λHHG5E

Nucleotides are numbered continuously from Glu^{56} of the H2A gene through to the 3' trailer region of the H2B gene. The locations of the H2A and H2B cap sites are marked by filled triangles at nt 227 and nt 459, respectively. The TATA-boxes and CCAAT-boxes for the H2A and H2B genes, as well as an OTF-1 site, are displayed in underlined bold font. The 3' untranslated region of the H2B gene contains the conserved histone 3' stem-loop motif,indicated by arrows and displayed in underlined bold font, as well as a poly(A) addition sequence located 1778 nt downstream from the protein stop codon, also displayed in underlined bold font. The 3' terminus of the 500 nt cell cycle dependent H2B mRNA is indicated by an empty triangle at nt 958 and the 3' terminus region of the 2222 nt cell cycle independent H2B mRNA is indicated by arrows at nt 2679-nt 2682. Consensus splice site sequences at nt 1010 and nt 2635 are illustrated by underlined bold font.

```
CTC GAG GAC CGC CGC CAT GTA GAC GGG CGC GCC GGC CCC CAC CCG CTC CGC GTA GTT GCC     60
Glu Leu Val Ala Ala Met Tyr Val Pro Ala Gly Ala Gly Val Arg Glu Ala Tyr Asn Gly

TTT GCG CAG CAA GCG GTG CAC TCG CCC TAC CGG GAA CTG GAG GCC AGC GCG GGA CGA GCG     120
Lys Arg Leu Leu Arg His Val Arg Gly Val Pro Phe Gln Leu Gly Ala Arg Ser Ser Arg

CGA CTT GGC CTT GGC GCG GGC CTT GCC TCC TTG TTT GCC ACG ACC AGA CAT     GACTGAAAT  180
Ser Lys Ala Lys Ala Arg Ala Lys Gly Gly Gln Lys Gly Arg Gly Ser Met

                                                          ←TATA-box
CAAGGTTAAA CACAGACAAC GCTCAGAACC TCGCAACAAA ATAAGATTGA AAGAGAACCA AGAGGAAAGC CTTTATAAGC  260
                          ←CCAAT-box                CCAAT-box→
TTTCCTAGGG GTGGGGTCAA GAACGAGTTT TTCATTGGTC CTAATATGGC TTCAGAACCG GCCAATCAAG TCAAGAGTCG  340
                                                                    OTF-1
GTGTTGTTAC GTACGCATTG CTGAAAACGC AAGGTCCGCA CACCGAATGG AAATGAACGA CTTTCGGAGC CCTAATTTGC  420
          TATA-box→
ATAAGGTGGT TATAAAAGAA TCAGGCCGCC ATTCTCTTAC TTCTTTTCTT GGCTAAGCCG CGTTTGTACT GTGTCTTACC  500

ATG CCT GAA CCG GCA AAA TCC GCT CCG GCG CCT AAA AAG GGC TCC AAG AAA GCC GTC ACC     560
Met Pro Glu Pro Ala Lys Ser Ala Pro Ala Pro Lys Lys Gly Ser Lys Lys Ala Val Thr

AAA GCC CAG AAG AAA GAC GGC AAG AAG CGC AAG CGC AGC CGC AAA GAG AGC TAC TCC ATC     620
Lys Ala Gln Lys Lys Asp Gly Lys Lys Arg Lys Arg Ser Arg Lys Glu Ser Tyr Ser Ile

TAC GTG TAC AAG GTG CTG AAG CAG GTC CAC CCC GAC ACC GGC ATC TCG TCC AAG GCC ATG     680
Tyr Val Tyr Lys Val Leu Lys Gln Val His Pro Asp Thr Gly Ile Ser Ser Lys Ala Met

GGC ATC ATG AAC TCC TTC GTC AAC GAC ATC TTC GAG CGC ATC GCG GGA GAG GCT TCC CGC     740
Gly Ile Met Asn Ser Phe Val Asn Asp Ile Phe Glu Arg Ile Ala Gly Glu Ala Ser Arg

CTG GCG CAC TAC AAC AAG CGC TCC ACC ATC ACA TCC CGC GAG ATC CAG ACG GCC GTG CGC     800
Leu Ala His Tyr Asn Lys Arg Ser Thr Ile Thr Ser Arg Glu Ile Gln Thr Ala Val Arg

CTG CTG CTG CCC GGC GAG CTG GCC AAG CAC GCC GTG TCC GAG GGC ACC AAG GCG GTC ACC     860
Leu Leu Leu Pro Gly Glu Leu Ala Lys His Ala Val Ser Glu Gly Thr Lys Ala Val Thr

AAG TAC ACC AGC TCC AAG TGA GTCCCTGCC GGGCACTGGC GCTCGCTCGC TCGAGTCGCC GGCTGCTTGA  930
Lys Tyr Thr Ser Ser Lys Stop

                ▽
CTCCAAAGGC TCTTTTCAGA GCCACCCACC TAATCACTAG AAAAGAGCTT GTTCACTTAT TCCCTTAGTT TCTTTTCATA  1010

AAGTAAGTTA TTTTAGTGTG AAGGTCATGG GAAATGGCAT ACGTAGCTTT TTAACTATTT GGAACTCGAG GTCCCCAGTG  1090

CGTCATTGGA TTTGCTTTTG AATCTAGAGC GTGTCTTTAC TCATTGTGCT GCTTAGCCTT CCCAGGAGTC GGTTCTCAAT  1170

TAGGCTGTTG GGAATCCGCC TCTTTACCCG CCCCCACTCC CGCCCCACAC GCGCCCTGGT GGCTCCTTGG GTTTGTTTCA  1250

TTCTAAAACG AAGTGGCTGA GTTCGGCTGT CATTTAAGAG AACTCCAGGA CACAATTCAG CCCGGGTTCC GCAAACACTG  1330
```

CGTGACAGCT CTGTATGACT GACGCTTGGC AGCAGCTTTT GTGTCCGGTC ACCAGTTCTG CCGTGCGATG GGGCCTCCTG 1410

TGGATACCAG CCGTTCTGTG TATTTTGGAC GAAGGCGGCA GCCGGGTCCC AGCCTTGTCC TGATTGGGCG ACAAGAATAT 1490

TCAAAATTCT GCGCCTTTTT CTAATTTGTA GATTTCAGTT TCCGTCGTTC ACTTTGAGAC TTTGAAATTC CTATTTCTCA 1570

TTTTGTTGAT AATTTCTGCA TTTAATGGTC TGTGCTTTAA ATGGTAACGC TACGGCCCCA GGTCACTGCG AGGCACTTAC 1650

CATGTAGATA CGGGCTCAAA AGTCACCTCT CAGAGACCTA CGTCATCCAC TCAGGAATTC GCGCCTCTCA TACTTGCCTG 1730

TCTCATTTTA TCTTCCTTCT AGCAGCTGTC TGAAATTGGT TCGTCTGTTT TCTTGTTTAT GGTATTCTCA AGCCCTTGAC 1810

AGACCGGCTA GTGTGGTTTT CCCGTGCATC TTCAGCCTGG CACATTATGG ACACTTAAAT ACTACGTATT GATCTAATAT 1890

TGTTGGGTTA ATTTTTCCAT CCCACCCTTT TCTTAATCGC TTCCGTGGAT GGATGAAGGG TGCTGTTCAT TTCCATTAGA 1970

TGTATGTGAA GGCACAGTGA AAATGGAAAT GTTCTTGGAG CTACTTCCTC AAAATGTATC CTTAGTCACC TCAGTGCAAC 2050

AGCTGGGAGG GGGCCGTGTT AAGATTTTTT TTGCTACAAA GAGGAGGTGG CAATGGTAGA TCCACCCTTA TGCTTGCTCA 2130

GTTTAGCATA ACCTCTTATG GATTTTCATC AAATTCAGCG TGTTGGTCAC TGGAAAGAGC CTTTTCCTTC TCCTTTTCTT 2210

ACTCTCCCCT CATGGTGTTC CCCTCTTAAA GGAGGAGGAG CTTTTAATTT ACACTTACCA CCTCATTTGC TTTTCTGGAG 2290

GCCATGCAAT ATAGGCGGGA CTACAGAGTT AATCTCCTTT TTACAAATGA GGCCAAGAGA AGCCTCATTG GTTCACAGTC 2370

ATGCAGCTCA TACTGTCCAC CCTTGTATTC TCAGATGCAG GACAATTGCA TTTTAGTTTT ATTTTGTGGA GGTGCAGAAT 2450

ATTTACTCTT TCTGTCCAAC CCTTGATTCT GCCGAGGAAG ACACTGATGG TTTGATGAGT GATTCAGCTG TTTTTGGCTA 2530

AGGGCTTTTG GAGCTGATGG CAGGGGTTTG ATGAATCCAA ATGAGCTCTA GACATTATCA CAGACTGAAT AGATCTTAAC 2610

TGTCTCCTAC ATGTGTGTTT TCAAATGTGT ATAGATGCTA TTGTTATTAA TAAAGTTACC AATTAATTTA AAGGCTTTGC 2690

TGGATTCTTT TATCTACAGT GTATTTTCTC AATGAATGAA ATTAACAGAT ACAACTTCCA CTGTGTCTGT AGAAAAGCGT 2770

GGGTGCCTCT GATCTGGGGG ATGACTATTT ATAAGCCACC AGCCAAACTG CTGCCCCAAA GGTGCACTGG GGCTGAAATA 2850

GTCTGTTACA AGGCCACTGT TAATGCTTAG TAATTGAAAA TATATGATCA CAATAGACAG CAGTACAATA GATGGAGAAT 2930

AAGTTCATAT AAGATCGTAA CACCACTAGA GCAC 2964

Figure 4-6 (continued)

H2B genes each contain consensus TATA (145) and CCAAT (42,177) boxes with the H2B promoter also including an OTF-1 element (50,70,92) (Figure 4-6). The H2B-GL105 gene includes a 3' non-translated region containing the conserved histone 3' stem-loop motif as well as a poly(A) addition sequence located 1778 nt downstream from the protein stop codon and consensus splice site sequences at nt 1010 and nt 2635 (Figure 4-6 and 4-7). Thus, this H2B.1 histone gene contains structural elements commonly found in cell cycle dependent histone genes as well as those reported for constitutively expressed histone genes. The H2B-GL105 gene is completely identical to the λHHC289 cDNA (35) and apparently contains no intervening sequences.

Genomic Organization of λHHG5E

To determine if apart from the H2A/H2B pair additional histone genes are located within the λHHG5E genomic segment, we carried out Southern blot analysis of Eco RI digested λHHG5E DNA (Figure 4-8) using ^{32}P-labeled probes for histones H1, H2A, H2B, H3 and H4 (See Materials and Methods section on Southern blot analysis for a detailed description of these histone probes). The results confirmed the presence of an H2A and an H2B histone gene within the 4100 nt Eco RI fragment of λHHG5E, but no additional histone genes were detected (Figure 4-8). However, this does not exclude the possibility that additional histone genes may be

Figure 4-7. Restriction data for the genomic clone λHHG5E

The locations of the H2A and H2B protein coding regions within the λHHG5E insert are illustrated by hatched boxes located within the 4100 nt Eco RI fragment. The 6100 nt Pst I fragment containing the entire H2B mRNA coding region and the conserved histone 3' stem-loop motif is expanded below the λHHG5E insert. Probe A used for S1 nuclease protection analysis is represented below the Pst I fragment. Restriction endonuclease sites are as follows: A, Asp 718; B, Bam HI; E, Eco RI; H, Hind III; N, Nco I; P, Pst I; S, Sac I; S', Sph I; X, Xho I.

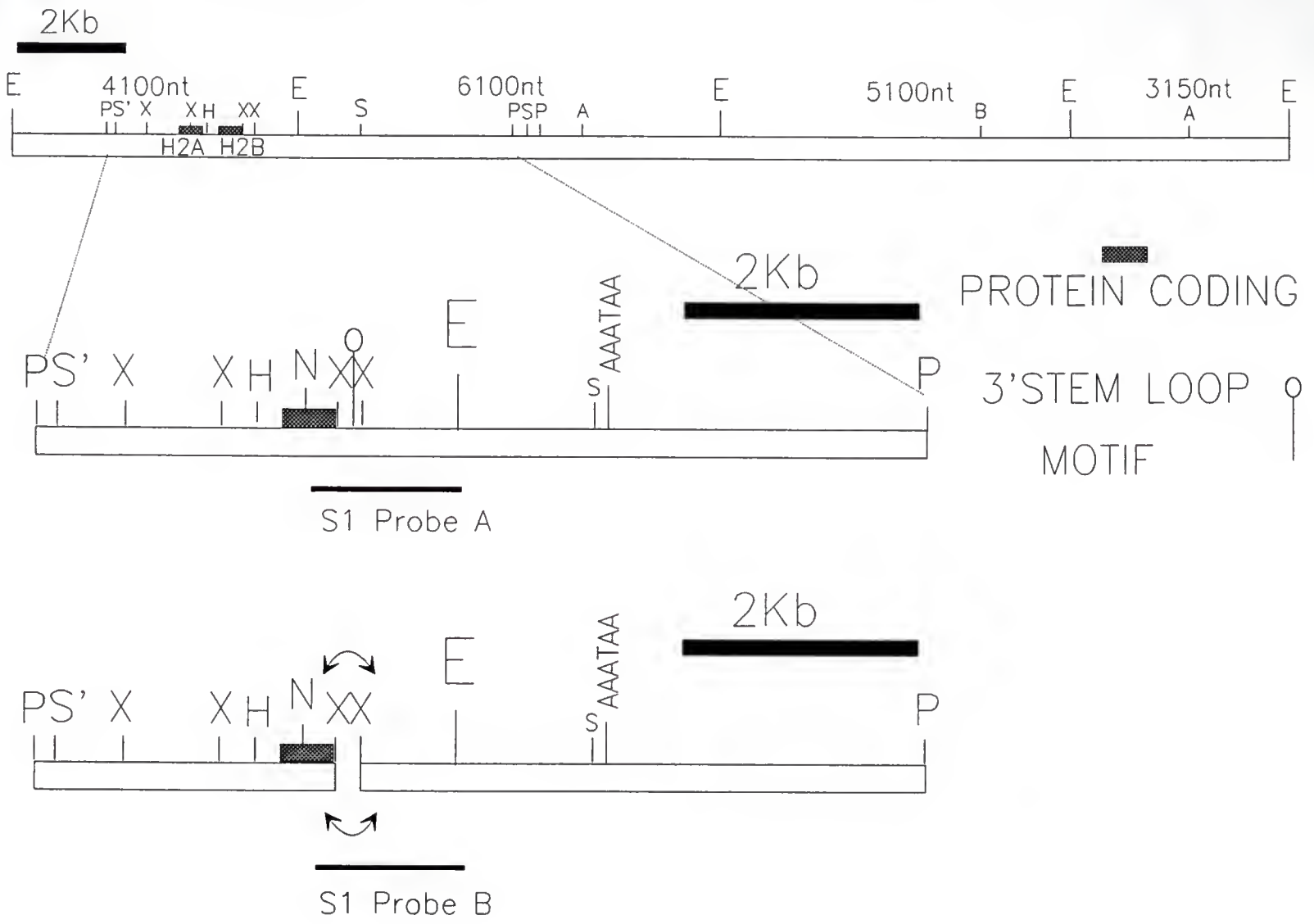
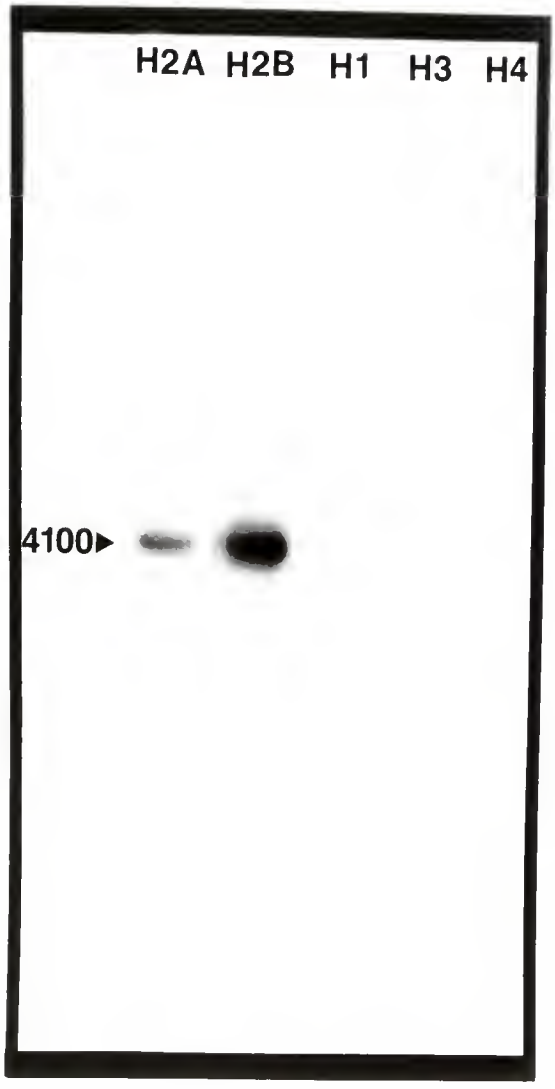

2Kb
E
4100nt
PS' X
X H
XX
H2A H2B
E
S
6100nt
PSP
A
E
5100nt
B
E
3150nt
A
E
2Kb
PROTEIN CODING
PS' X
X H
N
O
E
S
AAATAA
P
3'STEM LOOP
MOTIF
S1 Probe A
2Kb
PS' X
X H
N
XX
E
S
AAATAA
P
S1 Probe B

Figure 4-8. Southern blot analysis of the genomic clone λHHG5E with probes for histones H1, H2A, H2B, H3 and H4

Five lanes of a 0.8% agarose gel were loaded with 3.0 μg Eco RI restricted λHHG5E DNA each and the fragments separated electrophoretically. The DNA was transferred to Zeta-probe nylon membrane and each lane individually hybridized to either a histone H2A, H2B, H1, H3 or H4 ^{32}P-labeled DNA probe as indicated at the top of the figure (see Materials and Methods section on Southern blot analysis for a detailed description of the probes). The filled triangle indicates the position of the 4100 nt λHHG5E fragment containing the H2A and H2B genes.


H2A
H2B
H1
H3
H4
4100▶

within proximity but outside the boundaries of the λHHG5E genomic segment.

To evaluate if the H2B-GL105 gene is present as a single copy within the genome, we carried out Southern blot analysis of HeLa genomic DNA restricted with either Eco RI, Hind III or Pst I (Figure 4-9) using a ^{32}P-labeled probe (870 nt Eco RI/Sac I fragment isolated from λHHC289 (Figure 3-6) that spans the distal half of the H2B-GL105 3' non-translated trailer (Figure 1, nt 1706-nt 2577). As can be seen in Figure 3, the 3' non-translated probe hybridized to a single restriction fragment from each digest, indicating that it is present in a single copy in the human genome.

Chromosomal Localization of the H2B-GL105 Gene

To determine the chromosomal position of the H2B-GL105 gene, a panel of DNAs from rodent-human somatic cell hybrids carrying overlapping subsets of human chromosomes was tested for the presence of the H2B-GL105 locus by Southern blot analysis as shown in Figure 4-10. Hybridization of the Pst I digested human DNA revealed a 6100 nt Pst I fragment which was also detected in hybrids 77-31, Ph124 and BD3; mouse DNA and all other hybrid DNAs were negative for the H2B-GL105 locus. The three positive hybrids carry only human chromosomes 1 and X in common; many of the negative hybrids retain the human X chromosome; additionally all H2B-GL105 negative hybrids were also negative for

Figure 4-9. Southern blot analysis of HeLa genomic DNA with a H2B-GL105 probe

Each lane of a 0.8% agarose gel was loaded with 10 μg of HeLa genomic DNA restricted with: E, Eco RI; H, Hind III; P, Pst I. Lane M was loaded with 3'-labeled λ Eco RI/Hind III marker DNA. Following separation of the DNA fragments electrophoretically, the DNA was transferred to Zeta-probe nylon membrane and hybridized to a ^{32}P-labeled probe (870 nt Eco RI/Sac I fragment isolated from λHHC289 (see Figure 3-6)) that spans the distal half of the H2B-GL105 3' non-translated trailer (see Figure 4-6, nt 1706-nt 2577). The sizes of the marker fragments are indicated to the left of the autoradiogram in Kb.

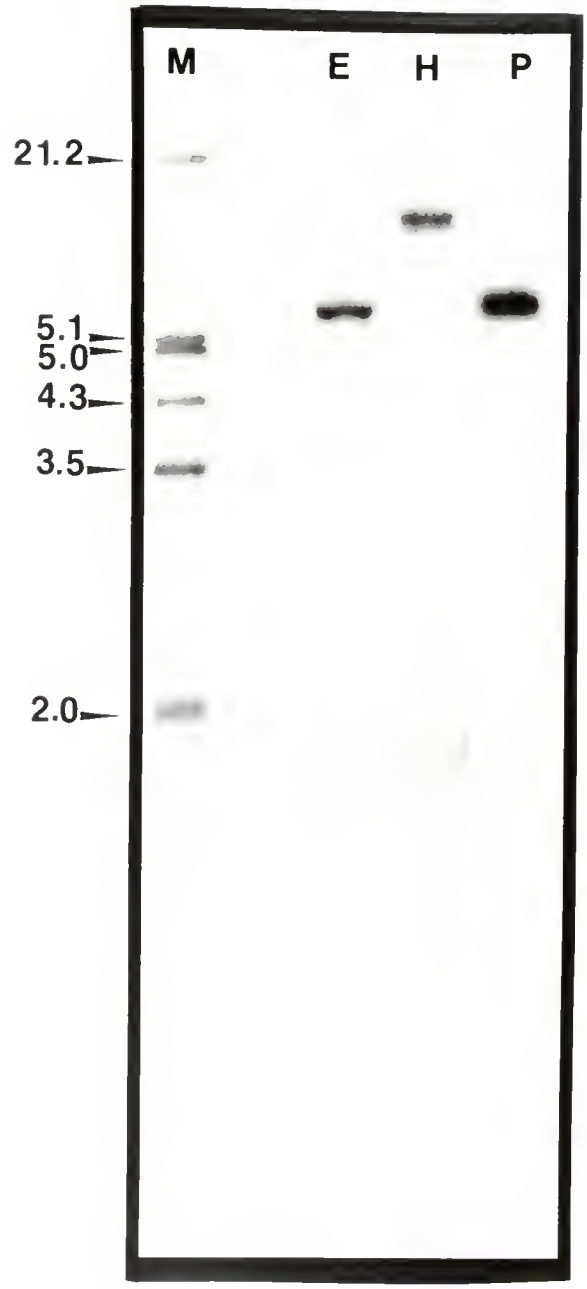

M
E
H
P
21.2
5.1
5.0
4.3
3.5
2.0

Figure 4-10. Southern blot analysis of DNAs from mouse-human somatic cell hybrids probed with a ^{32}P-labeled DNA fragment from the human histone gene H2B-GL105

An 0.8% agarose gel was loaded with ~10 μg/lane DNA from mouse (lane 1), human (lane 2), hybrid 8c (lane 3), hybrid PB5-1 (lane 4), hybrid 442S (lane 5), hybrid cl31 (lane 6), hybrid cl21 (lane 7), hybrid 77-31 (lane 8), hybrid cl17 (lane 9), hybrid Phl24 (lane 10), hybrid AA3 (lane 11), hybrid 3a (lane 12), hybrid N9 (lane 13), hybrid S3 (lane 14), hybrid AB3 (lane 15), hybrid C4 (lane 16), hybrid G5 (lane 17) and hybrid BD3 (lane 18) cleaved with Pst I, separated electrophoretically, transferred nylon membrane and hybridized to a ^{32}P-labeled probe (870 nt Eco RI/Sac I fragment isolated from λHHC289 (see Figure 3-6)) that spans the distal half of the H2B-GL105 3' non-translated trailer (Figure 4-6, nt 1706-nt 2577). Chromosomes retained by the hybrids are depicted in Figure 5.

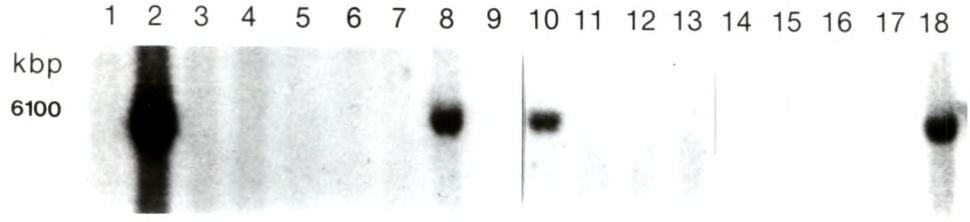
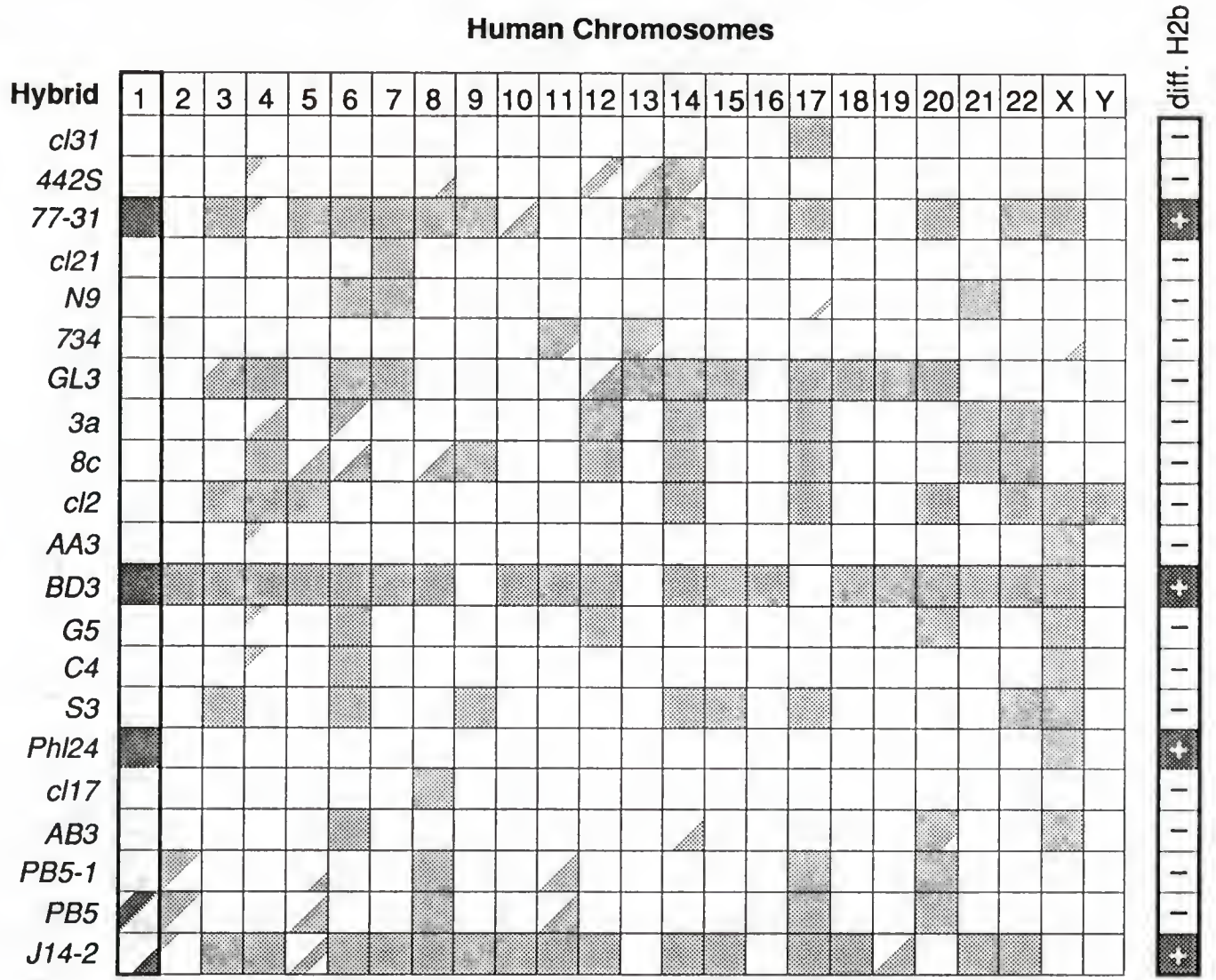

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
kbp
6100

Figure 4-11. Presence of the human histone gene H2B-GL105 in a panel of 21 rodent-human hybrids

A shaded box indicates that the hybrid named in the left column contains the chromosome indicated in the upper row; a shaded triangle in the lower right corner of a box indicates the presence of the long arm of the chromosome (or part of the long arm represented by an even smaller fraction of shading); a shaded triangle in the upper left corner of a box indicates the presence of the short arm (or partial short arm) of the chromosome; and an empty box indicates the absence of the chromosome listed above the column. The column for chromosome 1 is boldly outlined and stippled to highlight correlation of the presence of this chromosome, with presence of the H2B-GL105 gene. The pattern of retention of the H2B-GL105 gene in the panel is shown in the column to the right of the figure where the presence of the gene in the hybrid is indicated by a stippled box enclosing a plus sign and absence of the gene is indicated by an open box enclosing a minus sign.


Human Chromosomes
Hybrid
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
diff. H2b
cl31
442S
77-31
cl21
N9
734
GL3
3a
8c
cl2
AA3
BD3
G5
C4
S3
Phl24
cl17
AB3
PB5-1
PB5
J14-2
+
+
+

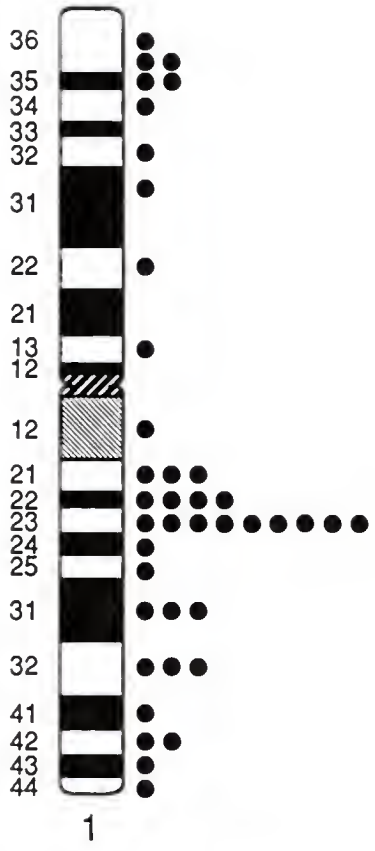
chromosome 1 (see Figure 4-11 for the chromosomal content of the hybrids). Thus, the presence of the H2B-GL105 histone gene in hybrid cells correlates with the presence of human chromosome 1 as summarized in Figure 4-11.

Additionally, the H2B-GL105 locus was absent in a hybrid, PB5, which retains most of the short arm (69) of chromosome 1 and was present in a hybrid, J14-2, which retains portions of the long arm of chromosome 1 but not the short arm (data not shown, but summarized in Figure 4-11). Thus, somatic cell hybrid analyses suggest localization of H2B-GL105 on the long arm of chromosome 1.

To confirm and refine the location of the H2B-GL105 gene on chromosome 1, in situ hybridization was carried out on metaphase chromosomes from a normal male subject (46, XY). A total of 100 metaphases were analyzed and 281 grains were hybridized to chromosomes. Of these, 11% (31/281) were hybridized selectively to the long arm of chromosome 1 (1q). The majority of grains on 1q were found within the q21-q23 subregion containing 61.3% (19/31) of the grains hybridized to 1q. Figure 4-12 illustrates the hybridization of the H2B-GL105 gene on chromosome 1 with the primary site of localization to 1q21-1q23. The in situ hybridization has, therefore, regionally localized the H2B-GL105 gene to the proximal region of 1q within q21-q23. These results coincide well with previously mapped histone

Figure 4-12. Regional localization of the human histone gene H2B-GL105 on human chromosome 1

The *in situ* hybridization of the H2B-GL105 probe to chromosome 1 is illustrated diagrammatically. The results indicate that 1q21-1q23 is the location of this gene.


36
35
34
33
32
31
22
21
13
12
12
21
22
23
24
25
31
32
41
42
43
44
1

genes. Several histones including H1, H3, and H4 also map to the 1q21 subregion (62).

H2B-GL105 mRNA Levels in Proliferating THP-1 Cells

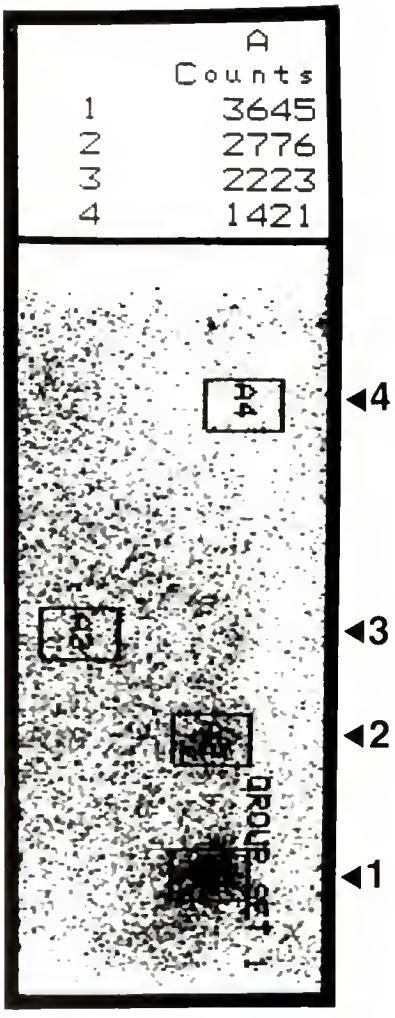
We have previously demonstrated that the H2B-GL105 gene expresses a 2300 nt cell cycle independent poly A+ mRNA containing consensus splice site sequences (chapter 3). These splice site sequences provide the cell with a potential mechanism by which alternative mRNAs may be expressed from a single gene. Alternatively, the conserved histone 3' stem-loop motif, present in the 3' non-translated region of the H2B-GL105 gene (Figure 1A and 1B), provides an additional avenue for the production of alternative mRNAs.

To determine if the H2B-GL105 gene expresses two mRNA species, we followed the mRNA levels of all H2B species in proliferating THP-1 cells by northern blot analysis using two different H2B ^{32}P-labeled probes. One probe was an H2B protein coding fragment from λHHC289 (Figure 4-13, lane A) and the second was a 58 nt fragment isolated from the H2B-GL105 5' non-translated leader (Figure 4-13, lane B). The protein coding probe cross hybridizes with all the H2B mRNAs in the cell and detects H2B mRNA species with sizes of 500 nt (band 1), 1100 nt (not well resolved from the strong 500 nt signal) and 2300 nt (band 2) (Figure 4-13, lane A). The 5' non-translated probe is specific for mRNA transcribed from the H2B-GL105 gene and detects 500 nt (band 1) and

Figure 4-13. Northern blot analysis of H2B-GL105 mRNA levels in proliferating THP-1 cells

Total cellular THP-1 RNA, 60 μg, was size fractionated, passively transferred to Zeta-probe nylon membrane, and hybridized to the ^{32}P-labeled 340 nt Eco RI/Xho I H2B protein coding fragment from λHHC289 (Figure 3-6) as probe (lane A). An identical northern analysis was carried out using the ^{32}P-labeled 58 nt Hae III/Nla III fragment (Figure 4-6, nt 446-nt 503) isolated from the H2B-GL105 5' non-translated leader (lane B). Lanes A and B contain results from autoradiography and lane C contains the results from a β-particle analysis (using a βetagen BetaScope 603 blot analyzer) of the lane B nylon membrane. In addition, the panel shown on the right side of the figure contains the numerical results of quantitative β-particle analysis of the hybridized membrane in lane B with reading 1 being of the 500 nt band, reading 2 being of the 2200-2300 nt band and readings 3 and 4 being of background regions of the nylon membrane.


A
B
C


A
Counts
1 3645
2 2776
3 2223
4 1421
◀4
◀3
◀2
◀1

2300 nt (band 2) H2B mRNA species (Figure 4-13, lanes B and C). The panel shown on the right side of Figure 4-13 contains the numerical results of quantitative β-particle analysis of the 500 nt band, the 2200-2300 nt band and background regions of the nylon membrane from the northern blot shown in lane C. The identity of the 2300 nt H2B mRNA species was verified by hybridization of this northern blot with a unique sequence probe specific for the 3' non-translated region of the H2B-GL105 gene. The results in Figure 4-13 demonstrate that the H2B-GL105 gene encodes a 500 nt H2B mRNA in addition to the 2300 nt mRNA previously described (chapter 3), with the 500 nt mRNA being more abundant than the 2300 nt species in proliferating THP-1 cells.

H2B-GL105 mRNA Levels in Synchronized HeLa Cells

To evaluate the cell cycle dependent nature of H2B-GL105 mRNAs, we carried out northern blot analysis on total cellular RNA isolated from synchronized HeLa cells after release from a double thymidine block and on samples enriched for poly A+ RNA using a single pass over oligo dT-cellulose. A single oligo dT-cellulose selection greatly enriches for poly A^+ RNA but does not totally remove all of the poly A^- RNA. Hybridization was carried out using a 58 nt, ^{32}P-labeled fragment isolated from the H2B-GL105 5' non-translated leader that is specific for H2B-GL105 mRNAs.

The 500 nt H2B-GL105 mRNA species was detected during the HeLa cell cycle (Figure 4-14) with its level reaching a maximum during the peak of DNA synthesis (4 hours). Despite having enriched for poly A+ RNA we were unable to detect the 2300 nt H2B-GL105 mRNA due to the small size of the hybridization probe and the low abundance of the 2300 nt H2B-GL105 mRNA (see chapter 3 and Figure 4-13). These results establish that the 500 nt H2B-GL105 mRNA species is regulated in a cell cycle dependent manner unlike the 2300 nt species which we have previously demonstrated to be constitutively expressed during the HeLa cell cycle (chapter 3).

Mapping of the H2B-GL105 5' mRNA Start Site

To determine the H2B-GL105 mRNA start site, S1 nuclease protection analysis was carried out on total cellular and poly A+ RNA using a 419 nt Hind III/Nco I probe derived from the H2B-GL105 gene (Figure 4-6, nt 258-nt 676) and 5' end labeled at the Nco I site (Figure 4-15). A 217 nt H2B protected fragment was detected, indicating the cap site for the H2B-GL105 mRNAs is located 42 nt upstream of the ATG translational start codon (Figure 4-15). The H2B fragment was detected primarily at the peak of S phase (4 hours) in the total cellular RNA sample (Figure 4-15); however, on longer exposure a protected fragment of the same size was observed outside of S phase and in the poly A+ RNA samples,

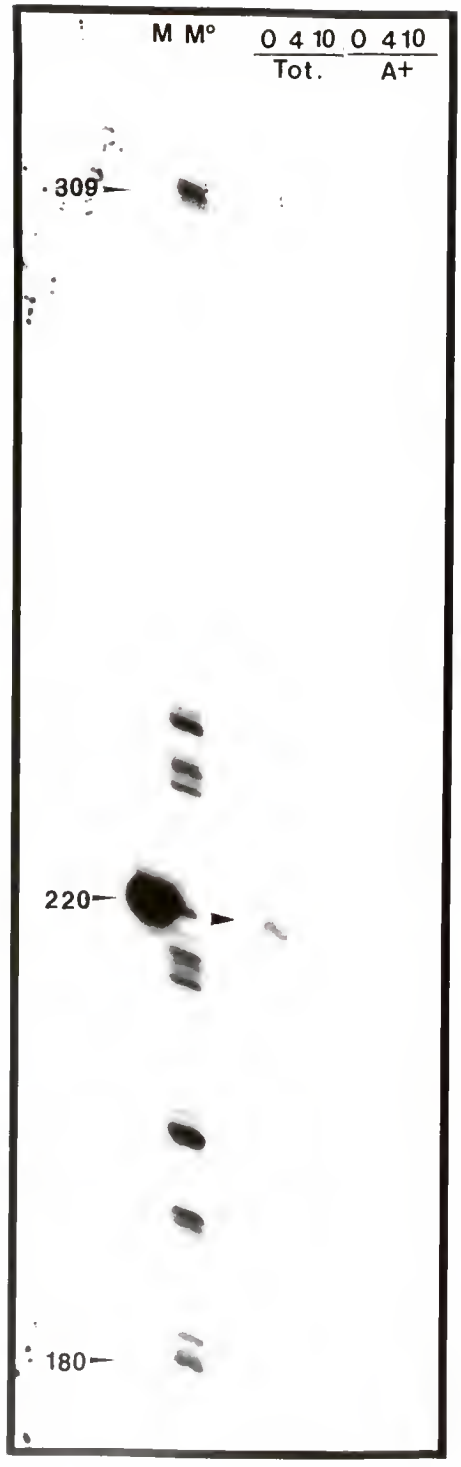
Figure 4-14. Northern blot analysis of H2B-GL105 mRNA levels in synchronized HeLa cells

Total cellular RNA was isolated from synchronized cells after (1, 2, 3, 4, 6, 8, and 10 hours) release from a double thymidine block and enriched for poly A+ RNA using a single pass over oligo dT-cellulose as described by Maniatis et al. (108). A single oligo dT-cellulose selection greatly enriches for poly A^+ RNA but does not totally remove all of the poly A^- RNA. The selected RNA, 3 μg, was size fractionated, passively transferred to Zeta-probe nylon membrane and hybridized to the ^{32}P-labeled 58 nt Hae III/Nla III fragment (Figure 4-6, nt 446-nt 503) isolated from the H2B-GL105 5' non-translated leader. The arrowhead at the left of the autoradiogram indicates the detected H2B-GL105 band.


1
2
3
4
6
8
10

Figure 4-15. S1 nuclease mapping of the H2B-GL105 5' mRNA start site

Total cellular and poly A+ RNAs were isolated from synchronized HeLa cells prior to (0) and after (4 and 10 hours) release from a double thymidine block. Total cellular RNA (Tot.), 50 μg, and 2 μg poly A+ RNA (A+) were used for S1 analysis. S1 analysis was carried out using a 419 nt Hind III/Nco I probe derived from the H2B-GL105 gene (Figure 4-6, nt 258-nt 676) and 5' end labeled at the Nco I site. Lanes M and M^0 were loaded with 3'-labeled pBR322 Hinf I and pBR322 Hpa II markers, respectively. The sizes of various marker bands are given on the left side of the figure. The 217 nt H2B protected fragment is indicated by an arrowhead in the center of the autoradiogram.


M M°
0 4 10 0 4 10
Tot. A+
309
220
180

demonstrating that the cap sites for both H2B-GL105 mRNAs are the same.

In addition, S1 nuclease protection analysis carried out on HeLa RNA, using a 257 nt Xho I/Hind III probe derived from the H2A-GL101 gene (Figure 4-6, nt 2-nt 258) and 5' end labeled at the Xho I site, produced a 225-226 nt H2A protected fragment, indicating that the cap site for the H2A-GL101 mRNAs is located 46 nt upstream of the ATG translational start codon (Figure 4-6). The S1 nuclease protection analysis clearly distinguished between correctly initiated H2A-GL101 transcripts and transcripts from other nonidentical H2A histone genes (Figure 4-16). The H2A protected fragment was detected primarily at the peak of S phase (4 hours).

Mapping of the H2B-GL105 mRNA 3' Termini

To investigate whether the 3' terminus of the 500 nt H2B-GL105 mRNA is typical of cell cycle dependent histone mRNAs, S1 nuclease protection analysis was carried out on total cellular (Figure 4-17, lane 1) and poly A+ (Figure 4-17, lane 2) RNA isolated from proliferating HeLa cells using a 3' end labeled probe derived from the λHHG5E H2B gene (Figure 4-7, S1 probe A). A 195 nt protected fragment mapping approximately to the H2B-GL105 stop codon and a 278 nt protected fragment mapping to the consensus histone mRNA terminus (12,166,167) were detected in the total

Figure 4-16. S1 mapping of the H2A-GL101 5' mRNA start site

Total cellular and poly A+ RNAs were isolated from synchronized HeLa cells prior to (0) and after (4 and 10 hours) release from a double thymidine block. Total cellular RNA (Tot.), 50 μg, and 2 μg poly A+ RNA (A+) were used for S1 analysis. S1 analysis was carried out using a 257 nt Xho I/Hind III probe derived from the H2A-GL101 gene (Figure 4-6, nt 2-nt 258) and 5' end labeled at the Xho I site. Lanes M and M° were loaded with 3'-labeled pBR322 Hinf I and pBR322 Hpa II markers, respectively. The sizes of various marker bands are given on the left side of the figure. Lanes A, C, G, and T were loaded with the products of dideoxy sequencing reactions on M13 single stranded template DNA (clone #16-3/1/90) utilizing a -40 primer (5'-GTTTTCCCAGTCACGAC-3'). The 225-226 nt H2A protected fragment is indicated by an arrowhead at the right of the autoradiogram.

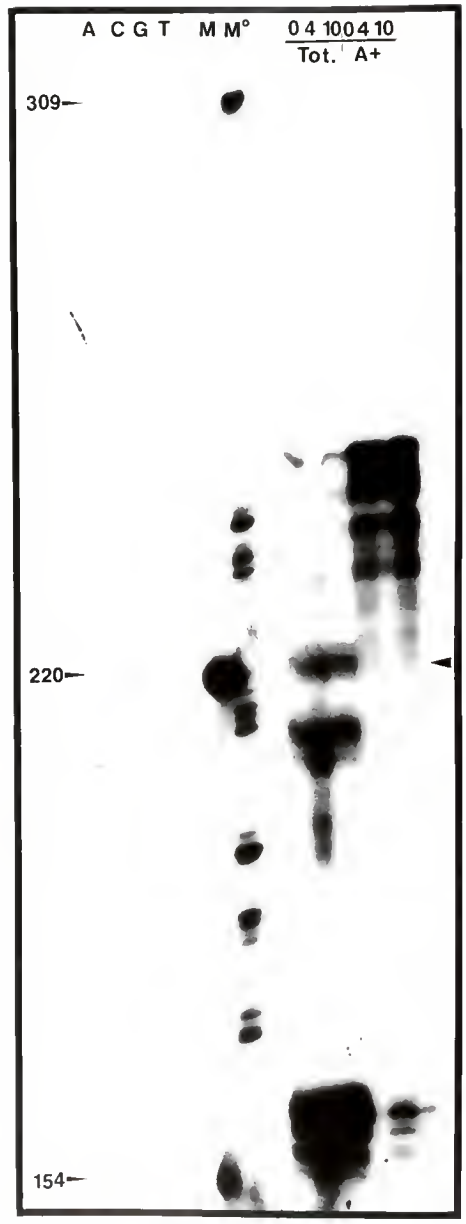
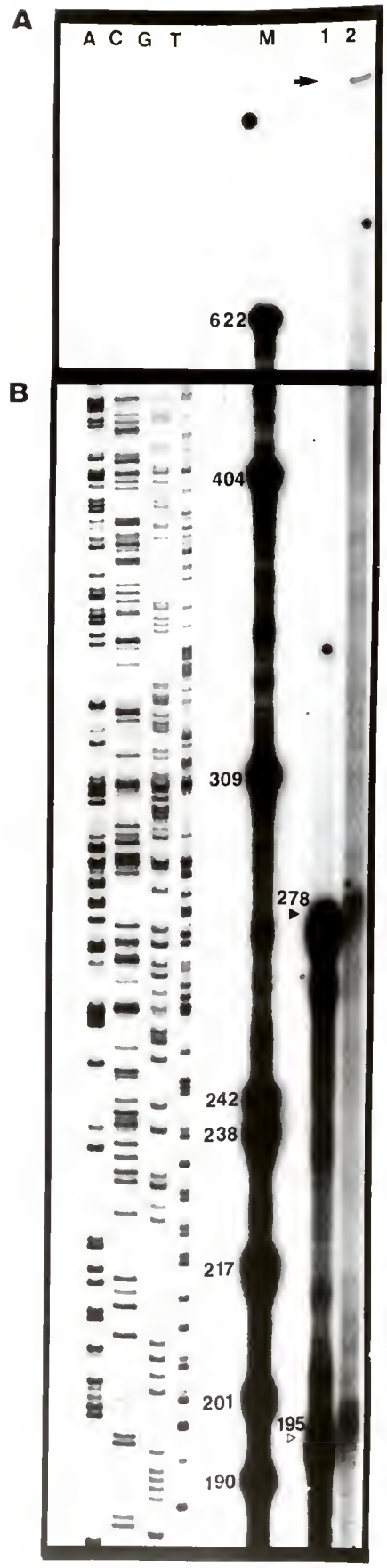

A C G T M M°
0 4 10 0 4 10
Tot. A+
309
220
154

Figure 4-17. S1 nuclease protection analysis of the 3' termini of the H2B-GL105 mRNA

Total cellular (lane 1) and poly A+ (lane 2) RNAs were isolated from proliferating HeLa cells. Total cellular RNA , 150 μg, and 4 μg poly A+ RNA were used for S1 analysis. S1 analysis was carried out using a 1028 nt Nco I/Eco RI probe derived from the H2B-GL105 gene (Figure 4-6, nt 681-nt 1028; Figure 4-7, S1 probe A) and 3' end labeled. Lane M was loaded with 3'-labeled pBR322 Hpa II markers. The sizes of various marker bands are given on the left side of lane M. Lanes A, C, G, and T were loaded with the products of dideoxy sequencing reactions on M13mp18 single stranded template DNA utilizing a -40 primer (5'-GTTTTCCCAGTCACGAC-3'). The 278 nt H2B-GL105 protected fragment is indicated by a filled triangle and the 195 nt H2B "stop codon" protected fragment is indicated by an empty triangle. The arrowhead at the top of the autoradiogram indicates protection to the end of S1 probe A (1028).


A
A C G T
M
1 2
622
B
404
309
278
242
238
217
201
195
190

cellular RNA sample (Figure 4-17, lane 1). In the poly A+RNA sample we observed a protected fragment corresponding to the 1028 nt probe, indicating protection to the end of S1 probe A (Figure 4-17, lane 2) which would be consistent with the presence of a 2300 nt poly A+ mRNA produced from the H2B-GL105 gene (see Figure 4-14 and chapter 3). Taken together these results verify the presence of two H2B-GL105 mRNA species and demonstrate that the 3' terminus of the 500 nt H2B-GL105 mRNA maps to the consensus histone mRNA terminus which is characteristic of cell cycle dependent histone mRNAs.

Discussion

Structure and Organization of the H2B-GL105 Gene

We have isolated and sequenced a variant human H2B histone gene (GL105) which expresses alternative mRNAs regulated differentially during the HeLa cell cycle. Our previous studies utilizing the H2B-GL105 cDNA (λHHC289) demonstrated that the 2300 nt H2B-GL105 mRNA species is reciprocally expressed in relation to replication-dependent H2B histone genes during HL60 cell differentiation (chapter 3). The nucleotide and deduced amino acid sequences reveal that the human H2B-GL105 gene encodes a protein identical to the somatic H2B.1 mouse protein in key amino acid positions (195). The H2B.1 histone variant is present in small amounts in rapidly growing tissues (195).

However, in those tissues with low growth rates in the adult, H2B.1 increases dramatically in relative amount during and especially just after the postnatal growth phase to reach a plateau at about 6 months (195). The cell must utilize complex transcriptional and post-transcriptional regulatory mechanisms to control cellular levels of mRNAs from various human H2B histone genes during progression through the cell cycle and at the onset of differentiation in order to modulate the ratios of H2B variant proteins in response to the growth state of the cell.

Transcriptional regulation of the H2B-GL105 Gene

Histone gene transcriptional regulation involves a transient increase in the rate of transcription at the G1/S phase boundary (113,151,163). Previously, nuclear run-on transcription analysis revealed a two fold increase in transcription of the H2B-GL105 gene during S phase (chapter 3). Sequence analysis shows that the H2B-GL105 gene contains, apart from a consensus TATA-box, a histone promoter specific CCAAT box (177) as well as an OTF-1 element (50) which are all promoter elements typical of cell cycle dependent H2B histone genes. The cell cycle transcription pattern of the H2B-GL105 gene and the apparent similarities of its promoter elements to those of cell cycle dependent H2B genes suggest that transcriptional regulation is not the sole modulator in controlling H2B variant ratios.

Post-Transcriptional Regulation of the H2B-GL105 Gene

We have observed that the H2B-GL105 gene expresses a 500 nt cell cycle dependent mRNA in addition to the 2300 nt cell cycle independent mRNA previously described (chapter 3). The 3' end of the cell cycle dependent mRNA terminates immediately following the region of hyphenated dyad symmetry, whereas the cell cycle independent mRNA has a 1798 nt non-translated trailer that contains the region of hyphenated dyad symmetry and a poly (A) addition sequence, followed by a poly (A) tail. The ability of the cell to utilize two mechanisms for mRNA 3' end formation may allow the H2B-GL105 gene to regulate the relative levels of H2B.1 protein in relation to cell cycle dependent histone protein during various growth states of the cell. However, it is unclear if the role of the H2B.1 protein is different than that of H2B.2 or if its purpose is solely to meet to limited need for histone protein at times when the majority of histone expression has been downregulated.

In proliferating cells we could detect very little H2B-GL105 mRNA in the poly A+ fraction, suggesting that the majority is processed to the 500 nt mature mRNA species using the endonucleolytic cleavage reaction observed by Birnstiel et al. (12). However, the level of the 2300 nt H2B-GL105 species increases approximately 10-fold during HL60 cell differentiation whereas the levels of cell cycle dependent H2B mRNAs decrease to less than 1% of those in

proliferating cells (chapter 3). This result would suggest that at the onset of differentiation the U7 mediated (166,167) histone maturation process is slowed or shut down, allowing additional time for polyadenylation of the histone pre-mRNA and resulting in an increase of the 2300 nt H2B-GL105 mRNA level.

In support of this suggestion, Hoffmann and Birnstiel have observed that 5' sequences of the U7 snRNP, which hybridize with the histone downstream spacer motif during 3' processing (147), are occluded in the G0 stage of the cell cycle (79), and Vasserot et al. have observed that during the transition from a proliferative to a nondividing state while rat myoblasts differentiate to myotubes the 5' ends of U7 snRNPs are protected against micrococcal nuclease attack (178) and presumably unavailable for histone 3' end processing. In addition, Lüscher and Schümperli have shown a histone 3' processing factor becomes limiting in G1-arrested cells (105), Stauber and Schümperli have demonstrated 3' processing of pre-mRNA plays a major role in proliferation-dependent regulation of histone mRNA levels (160), and Liu et al. have shown that the efficiency of 3' end formation contributes to the relative levels of different histone mRNAs (104). This mechanism may be operative for other histone genes which have been observed to produce multiple species of mRNA by alternative 3' end processing (27,29,94,110).

Alternatively, the 2300 nt mRNA may result from pre-mRNA transcripts being polyadenylated, due to the proximity of a poly A addition sequence to the stem-loop structure, before they are processed by the histone specific 3' endonucleolytic cleavage reaction. Such mRNA "slippage" would increase as a cell enters a nonproliferative state where factors such as the U7 snRNP and the heat labile factor, essential for cell cycle dependent histone mRNA 3' processing, become rate limiting. In such a mechanism, it would be unlikely that the protein product of this histone gene would differ functionally from cell cycle dependent H2B histone proteins.

In conclusion, we have characterized an H2B.1 gene which encodes both a 500 nt cell cycle dependent mRNA species and a 2300 nt constitutively expressed mRNA species which are regulated differentially in response to alterations in the proliferative state of the cell. Expression of these two mRNA species is regulated predominantly through alternative 3' end processing of the pre-mRNAs. These results suggest that 3' processing of histone H2B mRNA precursors may play an important role in the modulation of H2B.1 protein levels during the transition from a proliferative to a nondividing state.

CHAPTER 5

SUMMARY AND FUTURE CONSIDERATIONS

The initial emphasis of the research presented in this dissertation was to isolate and characterize a variant human histone gene and study its regulation in response to changes in the proliferative state of the cell. When this project was undertaken little was known concerning the structure or regulation of constitutively expressed human histone genes. Information in the literature on nonhuman variant histone genes showed these genes were structurally more complex than their replication-dependent counterparts, expressing polyadenylated mRNAs (46,71,96,100,116,143,144,175) which sometimes contained introns (20,43,185). In addition, Borun et al. observed that a fraction of human histone mRNAs contained short tracts of poly(A) (16). Early in the course of this project Wells and Kedes reported the cloning of a cDNA for a human H3.3 polyadenylated mRNA having intervening sequences (183).

Based on these observations, and our hypothesis that because constitutively expressed histone proteins are synthesized throughout the cell cycle (164,195) their mRNAs may be stable in the absence of DNA synthesis, we proposed

that structural features of constitutively expressed histone mRNAs may allow them to escape the mechanisms which rapidly and selectively destabilize replication-dependent histone mRNAs toward the end of S phase or in response to inhibition of DNA synthesis (3,76,117,142). Alternatively, the absence of structural elements commonly found in replication-dependent histone mRNAs could have the same result. Therefore, we focused our attention on the cloning of a variant human histone gene, and characterization of its structural features and their effect on expression in response to changes in the growth state of the cell.

In order to isolate a variant human histone gene, we first cloned and characterized a variant human histone cDNA (λHHC289) from a poly A+ cDNA library (chapter 3). λHHC289 contains an H2B protein coding region that is flanked at the 3' end by a 1798 nt non-translated trailer that includes a region of hyphenated dyad symmetry and a poly(A) addition sequence, followed by a poly(A) tail. We demonstrated that the HHC289 mRNA is regulated in a complex manner with respect to the HeLa cell cycle and HL60 cell differentiation. Northern blot analysis indicated that the levels of the 2300 nt HHC289 mRNA species did not vary significantly during the HeLa cell cycle, in comparison to replication-dependent H2B mRNAs which are elevated 15-fold higher during S phase than during G1 phase. Northern blot analysis also revealed a reciprocal relationship during the

onset of HL60 differentiation between the expression of the HHC289 H2B gene and the replication-dependent H2B genes. The levels of the 2300 nt HHC289 H2B species increased approximately 10-fold during HL60 cell differentiation whereas the levels of cell cycle dependent H2B mRNAs decreased to less than 1% of those in proliferating cells. Although we noticed the steady state level of HHC289 mRNA was regulated differentially to that of cell cycle dependent H2B histone mRNAs, which would be consistent with the dissimilar 3' structural features of their mRNAs, transcription analysis revealed that HHC289 transcription is cell cycle dependent during the HeLa cell cycle. These results suggest that complex post-transcriptional regulatory mechanisms control cellular levels of mRNAs from various human H2B histone genes during progression through the cell cycle and at the onset of differentiation.

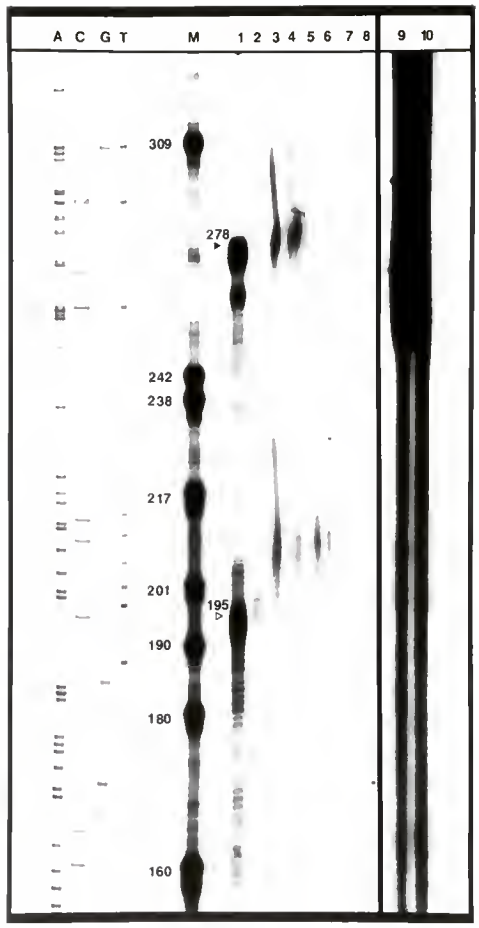
To further address the relationship between the structural features of the gene encoding the HHC289 H2B mRNA and their effect on expression in response to changes in the growth state of the cell we isolated this gene (H2B-GL105) from a human genomic DNA library. We demonstrated that H2B-GL105 expresses alternative mRNAs regulated differentially during the HeLa cell cycle, which is consistent with the presence of 3' structural elements commonly found in cell cycle dependent histone genes as well as those endogenous to constitutively expressed histone

genes. The 3' end of the cell cycle regulated mRNA terminates immediately following the region of hyphenated dyad symmetry typical of most histone mRNAs, whereas the constitutively expressed mRNA (HHC289) has a 1798 nt non-translated trailer that contains the same region of hyphenated dyad symmetry but is polyadenylated. These results demonstrate that alternative 3' end processing further increases the versatility by which cells can modulate the synthesis of replication-dependent as well as variant histone proteins during the cell cycle and at the onset of differentiation.

To begin to explore what roles the 3' stem-loop motif and the poly(A) addition sequence play in the regulation of H2B-GL105 mRNA levels and to determine if they are both essential for expression of the H2B-GL105 gene, we carried out a preliminary experiment examining the expression of various H2B-GL105 deletion mutants in 3T3L1 cells (Figure 5-1). We analyzed total cellular RNA from confluent and differentiated 3T3L1 cells, and cell lines established as described in materials and methods. S1 analysis was carried out on total cellular RNA isolated from confluent 3T3L1 cell lines 105-P7, 110-P10, 109-P7 and confluent 3T3L1 cells as well as differentiated 3T3L1 cell lines 105-P7, 110-P10, 109-P7 and differentiated 3T3L1 cells. In addition, S1 analysis was carried out on total cellular RNA and poly A+ RNA isolated from proliferating HeLa cells. Cell line

Figure 5-1. S1 analysis of the 3' termini of H2B mRNA expressed in 3T3L1 cell lines.

Total cellular (lane 1) and poly A+ (lane 2) RNAs were isolated from proliferating HeLa cells. In addition, total cellular RNA was isolated from confluent 3T3L1 cell lines 105-P7 (lane 4), 110-P10 (lane 6), 109-P7 (lane 10) and confluent 3T3L1 cells (lane 8) and differentiated 3T3L1 cell lines 105-P7 (lane 3), 110-P10 (lane 5), 109-P7 (lane 9) and, differentiated 3T3L1 cells (lane 7). Total cellular RNA, 150 μg, and 4 μg poly A+ RNA were used for S1 análysis. S1 analysis on all samples, with the exception of RNA samples isolated from the 3T3L1 cell line 109-P7, was carried out using a 1028 nt Nco I/Eco RI probe derived from the H2B-GL105 gene (Figure 4-6, nt 681-nt 1028; Figure 4-7, S1 probe A) and 3' end labeled, and these samples loaded in lanes 1-8. S1 analysis on RNA samples isolated from the 3T3L1 cell line 109-P7 was carried out using a 863 nt Nco I/Eco RI probe derived from the construct GL109SV (see Figure 2-5) which is similar to S1 probe A but with the 165 nt Xho I fragment deleted (Figure 4-7, S1 probe B). S1 probe B was 3' end labeled, and the 109-P7 samples were loaded in lanes 9 and 10. Lane M was loaded with 3'-labeled pBR322 Hpa II markers. The sizes of various marker bands are given on the left side of lane M. Lanes A, C, G, and T were loaded with the products of dideoxy sequencing reactions on M13mp18 single stranded template DNA utilizing a -40 primer (5'-GTTTTCCCAGTCACGAC-3'). The 278 nt H2B-GL105 protected fragment is indicated by a filled triangle and the 195 nt H2B "stop codon" protected fragment is indicated by an empty triangle.


A C G T
M
1 2 3 4 5 6 7 8
9 10
309
278
242
238
217
201
195
190
180
160

105-P7 is a control cell line containing the entire H2B-GL105 gene (Figure 2-2) and cell lines 109-P7 (Figure 2-5) and 110-P10 (Figure 2-4) contain deletions of the 3' stem-loop motif and poly(A) addition sequence, respectively. A 195 nt protected fragment mapping approximately to the H2B-GL105 stop codon and a 278 nt protected fragment mapping to the consensus histone mRNA terminus (12,166,167) were detected in the total cellular RNA samples for HeLa and 3T3L1 cell line 105-P7 (Figure 5-1, lanes 1,3, and 4). However, only the 195 nt protected fragment (an H2B stop codon protection) was detected for the cell line 110-P10 and the 3T3L1 control sample (Figure 5-1, lanes 5, 6, 7, and 8) indicating the presence of H2B histone mRNAs in these samples but none specific for the GL110SV construct (Figure 2-4). The S1 analysis of the RNA from the 109-P7 cell line was uninterpretable (Figure 5-1, lanes 8 and 9); a continuous signal was detected throughout the length of the gel, even with a shorter exposure. Similar results were obtained in transient expression assays using RNA isolated 48 hours after transfection of 3T3L1 cells with the GL109SV construct (data not shown). These data indicate that the 105-P7 cell line expresses the H2B-GL105 mRNA whereas the 110-P10 mRNA does not accumulate to detectable levels in the 110-P10 cell line. The results for the 109-P7 cell line are uninterpretable. Although these results are preliminary, they do suggest that deleting a portion of the 3' trailer of

the H2B-GL105 gene containing the poly(A) addition sequence (construct GL110SV, Figure 2-4) alters the expression of, or 3' processing of, the H2B-GL110SV mRNA resulting in no detectable accumulation of H2B-GL110SV mRNA. Additional research may lead to a better understanding of the role that the 3' stem-loop motif and the poly(A) addition sequence play in the regulation of H2B-GL105 mRNA. A systematic analysis of constructs containing deletions throughout the 3' trailing region of the H2B-GL105 gene may provide insight into what sequences are essential for expression of stable replication dependent and constitutively expressed H2B-GL105 mRNAs. In addition, such analyses may reveal whether sequences in close proximity to the 3' stem-loop motif are sufficient for expression of a replication dependent H2B-GL105 mRNA or whether additional sequences further downstream are required.

In summary, the work presented in this dissertation demonstrates that the variant H2B-GL105 gene expresses alternative mRNAs whose processing is dependent upon structural elements present in the 3' trailer (such as the 3' stem-loop motif and poly (A) addition sequence). The H2B-GL105 gene has 3' structural features inherent to both cell cycle dependent and constitutively expressed H2B genes and thus allows for the expression of its H2B.1 gene product during the cell cycle as well as at the onset of differentiation. The expression of alternative mRNAs by the

H2B-GL105 gene provides the cell with the versatility to modulate the synthesis of replication-dependent H2B proteins during the transition from a proliferative to a nondividing state.

APPENDIX A

REAGENTS FOR PROPAGATION AND MAINTENANCE OF BACTERIA AND BACTERIOPHAGE

1. Ampicillin, 10 mg/ml

One gram of the sodium salt of ampicillin was dissolved in 80 ml ddH_2O, the pH of the solution adjusted to 7.4 by adding 1 M HCl dropwise and the volume brought to 100 ml. The solution was sterilized by filtration and divided into 10 ml aliquots which were stored at -20°C. The working concentration of ampicillin used was 50 μg/ml.

Ampicillin kills only growing bacteria and functions by inhibiting cell wall synthesis by blocking formation of the peptidoglycan cross-link (reviewed by Davies and Smith (41); Waxman and Strominger (181)).

2. Chloramphenicol, 20 mg/ml

Two grams of chloramphenicol were dissolved in 100 ml 95% ethanol and stored at -20°C. For amplification of plasmids the working concentration of chloramphenicol used was 200 μg/ml.

Chloramphenicol inhibits protein synthesis in bacteria by binding to the 50S ribosomal subunit and inhibiting the peptidyltransferase reaction (reviewed by Davies and Smith (41)).

3. Glucose, 20% (100X)

Twenty grams of glucose were dissolved in ddH_2O and the volume brought to 100 ml. The solution was sterilized by filtration and stored at 4°C.

4. Maltose, 20% (100X)

Twenty grams of maltose were dissolved in ddH_2O and the volume brought to 100 ml. The solution was sterilized by filtration and stored at 4°C.

5. NZCYM Medium

Component	Per liter
NZ amine	10.0 g
NaCl	5.0 g
casamino acids	1.0 g
Bacto-yeast extract	5.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g

Components were dissolved in ddH_2O bringing the solution to 80% final volume and the pH adjusted to 7.4 by adding 1 N NaOH dropwise. The volume was next brought to 105% of the desired final volume and the medium sterilized by autoclaving.

6. NZCYM Bottom Agar Plates

Component	Per liter
Bacto-agar	15.0 g
NZCYM medium	1.0 l

Components were added to an erlenmeyer flask and the suspension was sterilized by autoclaving. After removal from the autoclave the flask was carefully swirled and then cooled to 50°C in a water bath. After cooling, the bottom agar was poured into disposable plastic petri dishes and stored at 4°C.

7. NZCYM Top Agar

Component	Per liter
Bacto-agar	7.0 g
NZCYM medium	1.0 l

Components were added to an erlenmeyer flask and the suspension was sterilized by autoclaving. After removal from the autoclave the flask was carefully swirled and then cooled to 50°C in a water bath. After cooling, the top agar was divided into 4.0 ml aliquots in culture tubes, the caps sealed with parafilm to prevent moisture loss and stored at room temperature. Prior to use, the top agar was melted by placing the tubes in a 95°C water bath for 10-15 minutes and then cooled to 45°C before use.

8. SM Medium

Component	Per liter
NaCl	5.8 g
$MgSO_4 \cdot 7H_2O$	2.0 g
1 M Tris•Cl (pH 7.5)	50.0 ml
2% gelatin	5.0 ml

Components were dissolved in ddH_2O bringing the solution to 80% final volume and the pH adjusted to 7.4 by adding 1 N NaOH dropwise. The volume was next brought to 105% of the desired final volume the medium sterilized by and autoclaving.

9. Tetracycline 12.5 mg/ml

Tetracycline hydrochloride (1.25 g) was dissolved in 100 ml ethanol/water (50% v/v) and stored at -20°C. Ethanol/water (50% v/v) was prepared by mixing 53 ml 95% ethanol and 47 ml ddH_2O. The working concentration of tetracycline used was 15 μg/ml. Tetracycline is light-sensitive so solutions and plates containing the antibiotic were wrapped in foil for storage.

Magnesium ions are antagonists of tetracycline so media containing magnesium salts (e.g. NZCYM) were not used when tetracycline was required.

Tetracycline inhibits bacterial protein synthesis by binding to the 30S subunit of the ribosome which prevents binding of aminoacyl tRNA to the ribosome A site thereby,

inhibiting ribosomal translocation (reviewed by Davies and Smith (41)).

10. YTN Medium

Component	Per liter
Bacto-tryptone	8.0 g
Bacto-yeast extract	10.0 g
NaCl	5.0 g

Components were dissolved in ddH_2O bringing the solution to 80% final volume and the pH adjusted to 7.4 by adding 1 N NaOH dropwise. The volume was next brought to 105% of the desired final volume and the medium sterilized by autoclaving.

11. YTN Bottom Agar Plates

Component	Per liter
Bacto-agar	15.0 g
YTN medium	1.0 l

Components were added to an erlenmeyer flask and the suspension was sterilized by autoclaving. After removal from the autoclave the flask was carefully swirled and then cooled to 50°C in a water bath. After cooling, the bottom agar was poured into disposable plastic petri dishes and stored at 4°C. If necessary, ampicillin was added to the YTN agar to a final concentration of 50 μg/ml or

tetracycline to 15 μg/ml immediately prior to pouring of the plates.

12. YTN Top Agar

Component	Per liter
Bacto-agar	7.0 g
YTN medium	1.0 l

Components were added to an erlenmeyer flask and the suspension was sterilized by autoclaving. After removal from the autoclave the flask was carefully swirled and then cooled to 50°C in a water bath. After cooling, the top agar was divided into 4.0 ml aliquots in culture tubes, the caps sealed with parafilm to prevent moisture loss and stored at room temperature. Prior to use, the top agar was melted by placing the tubes in a 95°C water bath for 10-15 minutes and then cooled to 45°C before use.

13. YTN Top Agarose

YTN top agarose was prepared essentially as described for YTN top agar but with agarose substituting for agar. Top agarose provides a firmer surface for lifting nitrocellulose filters from the plates during bacteriophage screening.

Appendix B

REAGENTS FOR NUCLEIC ACID ISOLATION AND PURIFICATION

1. Phenol, Equilibrated

Phenol was redistilled at 160°C, 8-Hydroxyquinoline added to a final concentration of 0.1% and stored in 500 ml aliquots at -20°C under nitrogen gas. Aliquots of phenol were removed from the freezer and melted in a 65°C water bath. The melted phenol was then extracted once with a 50% volume of 1 M Tris•Cl (pH 8.0) followed by several equal volume extractions with 10 mM Tris•Cl (pH 8.0), 1 mM EDTA until the pH was approximately 8.0. The equilibrated phenol was then stored at 4°C under 10 mM Tris•Cl (pH 8.0), 1 mM EDTA in a brown, glass bottle.

2. Alkaline Lysis Solution #1

Component	Per liter	Final concentration
20% α-D Glucose	45 ml	0.9% (w/v)
1 M Tris•Cl (pH 8.0)	25 ml	25 mM
500 mM EDTA	20 ml	10 mM

Components were combined and the solution brought to 1.0 liter final with the addition of 910 ml ddH_2O.

3. Alkaline Lysis Solution #2

Component	Per 100 ml	Final concentration
20% SDS (w/v)	5 ml	1.0% (w/v)
5 M NaOH	4 ml	200 mN

The 5 M NaOH stock, 4 ml, was added to 91 ml ddH_2O and mixed thoroughly. Next the 20% SDS (w/v) stock, 5 ml, was added and the solution mixed. Alkaline lysis solution #2 was made fresh for each plasmid preparation.

Appendix C

BACTERIAL STRAINS USED

Strain	Remarks	Reference
DH5α	Suitable for generating cDNA libraries and for other plasmid cloning procedures.	BRL™ (8)
LE392	LE392 was prepared by L. Enquist and is a derivative of strain ED8654.	Borck et al. (15) Murray et al. (121) Enquist et al. (44)
XL1-Blue	Stability of the F' episome is provided by the tetracycline resistant marker located on the F' episome.	Bullock et al. (21)
Y1088	Y1088 was derived from KM392 (itself a derivative of LE392) by transformation with plasmid pMC9, a pBR322 plasmid carrying lac I.	Young and Davis (191)

REFERENCES

1. **Allfrey, V.G., V.C. Littau, and A.E. Mirsky.** 1963. On the role of histones in regulating ribonucleic acid synthesis in the cell nucleus. Proc. Natl. Acad. Sci. USA **49**:414-421.

2. **Alterman, R-B.M., C. Sprecher, R. Graves, W.F. Marzluff, and A.I. Skoultchi.** 1985. Regulated Expression of a chimeric histone gene introduced into mouse fibroblasts. Mol. Cell. Biol. **5**:2316-2324.

3. **Baumbach, L.L., F. Marashi, M. Plumb, G. Stein, and J. Stein.** 1984. Inhibition of DNA replication coordinately reduces cellular levels of core and H1 histone mRNAs; requirement for protein synthesis. Biochemistry **23**:1618-1625.

4. **Baumbach, L.L., G.S. Stein, and J.L. Stein.** 1987. Regulation of human histone gene expression: transcriptional and post transcriptional control in the coupling of histone messenger RNA stability with DNA replication. Biochemistry **26**:6178-6187.

5. **Benton, W.D., and** R.W. Davis. 1977. Screening λgt recombinant clones by hybridization to single plaques in situ. Science **196**:180-182.

6. **Berk, A.J., and P.A.** Sharp. 1978. Spliced early mRNAs of simian virus 40. Proc. Natl. Acad. Sci. USA **75**:1274-1278.

7. **Bernlohr, D.A., C.W.** Angus, **M.D. Lane, M.A. Bolanowski, and T.J. Kelly,Jr..** 1984. Expression of specific mRNAs during adipose differentiation: Identification of an mRNA encoding a homologue of myelin P2 protein. Proc. Natl. Acad. Sci. USA **81**:5468-5472.

8. **Bethesda Research** Laboratories 1988. Catalogue & reference guide. Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, MD.

9. **Birchmier, C., R. Grosschedl, and M.L. Birnstiel.** 1983. Generation of authentic 3' termini of an H2A in vivo is dependent on a short inverted DNA repeat and on spacer sequences. Cell **28**:739-745.

10. **Bird, R.C., F.A. Jacobs, G. Stein, J. Stein, and B.H. Sells.** 1985. Coordinate regulation of histone mRNAs during growth and differentiation of rat myoblasts. Biochim. Biophys. Acta **824**:209-217.

11. **Birnboim, H.C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513-1523.

12. **Birnstiel, M.L., M. Busslinger, and K. Strub.** 1985. Transcription termination and 3' processing: the end is in site. Cell **41**:349-359.

13. **Blattner, F.R., B.G. Williams, A.E. Blechl, K. Denniston-Thompson, H.E. Faber, L. Furlong, D.J. Grunwald, D.O. Kiefer, D.D. Moore, J.W. Schumm, E.L. Sheldon, and O. Smithies.** 1977. Charon phages: Safer derivatives of bacteriophage lambda for DNA cloning. Science **196**:161-169.

14. **Blin, N., and D.W. Stafford.** 1976. A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res. **30**:2303.

15. **Borck, K., J.D. Beggs, W.J. Brammar, A.S. Hopkins, and N.E. Murray.** 1976. The construction in vitro of transducing derivatives of phage lambda. Molec. gen. Genet. **146**:199-207.

16. **Borun, T.B., K. Ajiro, A. Zweidler, T.W. Dolby, and R.E. Stephenns.** 1977. Studies of human histone messenger RNA. J. Biol. Chem. **252**:173-180.

17. **Branson, R.E., S.R. Grimes, G. Yonuschot, and J.L. Irvin.** 1975. The histones of rat testis. Arch. Biochem. Biophys. **168**:403-412.

18. **Breathnach, R., and P. Chambon.** 1981. Organization and expression of eucaryotic split genes coding for proteins. Annu. Rev. Biochem. **50**:349-383.

19. **Brown, D.T., S.E. Wellman, and D.B. Sittman.** 1985. Changes in the levels of three different classes of histone mRNA during murine erythroleukemia cell differentiation. Mol. Cell. Biol. **5**:2879-2886.

20. **Brush, D., J.B. Dodgson, O.-R. Choi, P.W. Stevens, and J.D. Engel.** 1985. Replacement variant histone genes contain intervening sequences. Mol. Cell. Biol. **5**:1307-1317.

21. **Bullock, W.O., J.M.** Fernandez, **and J.M. Short.** 1987. XL1-Blue: A high efficiency plasmid transforming recA Escherichia coli strain with beta-galactosidase selection. Biotechniques **5**:376-379.

22. **Butler, W.B., and G.C. Mueller.** 1973. Control of histone synthesis in HeLa cells. Biochim. Biophys. Acta **294**:481-496.

23. **Cannizzaro, L.A.,** P. Madaule, **F. Hecht, R. Axel, C.M. Croce, and K. Huebner.** 1990. Chromosome localization of human ARH genes, a ras-related gene family. Genomics **6**:197-203.

24. **Carozzi, N.,** F. Marashi, M. **Plumb, S. Zimmerman, A. Zimmerman, L.S. Coles,** J.R.E. Wells, **G. Stein, and J. Stein.** 1984. Clustering of human H1 and core histone genes. Science **224**:1115-1117.

25. **Carrino, J.J.,** V. Kueng, R. **Braun, and T.G. Laffler.** 1987. Distinct replication-independent and -dependent phases of histone gene expression during the Physarum cell cycle. Mol. Cell. Biol. 7:1933-1937.

26. **Chaconas, G., and** J.H. van **de Sande.** 1980. 5'-^{32}P Labeling of RNA and DNA restriction fragments. Meth. Enzymol. **65**:75-85.

27. **Challoner, P.B.,** S.B. Moss, **and M. Groudine.** 1989. Expression of replication-dependent histone genes in avian spermatids involves an alternate pathway of mRNA 3' end formation. Mol. Cell. Biol. 9:902-913.

28. **Chaubet, N.,** M. Chaboute, B. **Clément, M. Ehling, G. Philipps, and C. Gigot.** 1988. The histone H3 and H4 mRNAs are polyadenylylated in maize. Nucleic Acids Res. **16**:1295-1304.

29. **Cheng, G., A. Nandi,** S. Clerk, **and A.I. Skoultchi.** 1989. Different 3'-end processing produces two independently regulated mRNAs from a single H1 histone gene. Proc. Natl. Acad. Sci. USA **86**:7002-7006.

30. **Cheng, G., and A.I.** Skoultchi. 1989. Rapid induction of polyadenylated H1 histone mRNAs in mouse erythroleukemia cells is regulated by c-myc. Mol. Cell. Biol. **9**:2332-2340.

31. **Chodchoy, N.,** B.J. Levine, **C. Sprecher, A.I. Skoultchi, and W.F. Marzluff.** 1987. Expression of mouse histone genes: transcription into 3' intergenic DNA and cryptic processing sites downstream from the 3' end of the H3 gene. Mol. Cell. Biol. **7**:1039-1047.

32. **Clayton, D., and J. Darnell Jr.**. 1983. Changes in liver-specific compared to common gene transcription during primary culture of mouse hepatocytes. Mol. Cell. Biol. **3**:1552-1561.

33. **Clewell, D.B., and D.R. Helinski.** 1970. Properties of a supercoiled deoxyribonucleic acid-protein relaxation complex and strand specificity of the relaxation event. Biochemistry **9**:4428-4440.

34. **Cole, K.D., J.C. Kandala, and W.S. Kistler.** 1986. Isolation of the gene for the testis-specific H1 histone variant H1t. J. Biol. Chem. **261**:7178-7183.

35. **Collart, D., A. Ramsey-Ewing, R. Bortell, J. Lian, J. Stein, and G. Stein.** 1991. Isolation and characterization of a cDNA from a human histone H2B gene which is reciprocally expressed in relation to replication-dependent H2B histone genes during HL60 cell differentiation. Biochemistry (In Press)

36. **Collart, D., G.S. Stein, and J.L. Stein.** 1985. A series of repetitive DNA sequences are associated with human core and H1 histone genes. Mol. Cell. Biochem. **67**:161-170.

37. **Collart, D.G., K.L. Wright, A.J. van Wijnen, A.L. Ramsey, J. Lian, J.L. Stein, and G.S. Stein.** 1988. The human H1 histone gene FNC16 is functionally expressed in proliferating HeLa S3 cells and is down-regulated during terminal differentiation in HL60 cells. J. Biol. Chem. **263**:15860-15863.

38. **Cosenza, S.C., T.A. Owen, D.R. Soprano, and K.J. Soprano.** 1988. Evidence that the time of entry into S is determined by events occurring in early G1. J. Biol. Chem. **263**:12751-12758.

39. **Cotten, M., O. Gick, A. Vasserot, G. Schaffner, and M.L. Birnstiel.** 1988. Specific contacts between mammalian U7 snRNA and histone precursor RNA are indispensable for the *in vitro* 3' RNA processing reaction. EMBO J. **7**:801-808.

40. **Dalton, S., J.R. Coleman, and J.R.E. Wells.** 1986. Transcription of the histone H5 gene is not S-phase regulated. Mol. Cell. Biol. **6**:601-606.

41. **Davies, J., and** D.I. Smith. 1978. Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. **32**:469-518.

42. **Efstratiadis, A., J.W. Posakony, T. Maniatis, R.M. Lawn, Connell,C. O, R.A. Spritz, J.K. DeRiel, B.G. Forget, S.M. Weissman, J.L. Slightom, A.E. Blechl, O. Smithies, F.E. Baralle, C.C. Shoulders, and N.J. Proudfoot.** 1980. The structure and evolution of the human β-globin gene family. Cell **21**:653-668.

43. **Engel, J.D., B.J. Sugarman, and J.B. Dodgson.** 1982. A chicken histone H3 gene contains intervening sequences. Nature **297**:434-436.

44. **Enquist, L.W., and A. Skalka.** 1973. Replication of bacteriophage λ DNA dependent on the function of host and viral genes. J. Mol. Biol. **75**:185-212.

45. **Ernst, S.G., H. Miller, C.A. Brenner, C. Nocente-McGrath, S. Francis, and R. McIsaac.** 1987. Characterization of a cDNA clone coding for a sea urchin histone H2A variant related to the H2A.F/Z histone protein in vertebrates. Nucleic Acids Res. **15**:4629-4644.

46. **Fahrner, K., J. Yarger, and L. Hereford.** 1980. Yeast histone mRNA is polyadenylated. Nucleic Acids Res. **8**:5725-5737.

47. **Feinberg, A.P., and Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137**:266-267.

48. **Feinberg, A.P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6-13.

49. **Fitzgerald, M., and T. Shenk.** 1981. The sequence 5'-AAUAAA-3' forms part of the recognition site for polyadenylation of late SV40 mRNAs. Cell **24**:251-260.

50. **Fletcher, C., N. Heintz, and R.G. Roeder.** 1987. Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. Cell **51**:773-781.

51. **Flint, S.J., M.A. Plumb, U.-C. Yang, G.S. Stein, and J.L. Stein.** 1984. Effect of adenovirus infection on expression of human histone genes. Mol. Cell. Biol. **4**:1363-1371.

52. **Fort, Ph., L. Marty, M. Piechaczyk, S.E. Sabrouty, Ch. Dani, Ph. Jeanteur, and J.M. Blanchard.** 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res. **13**:1431-1442.

53. **Friedman, D.L., and M.J. Imperiale.** 1987. RNA 3' end formation in the control of gene expression. Annu. Rev. Genet. **21**:453-488.

54. **Frischauf, A-M., H. Lehrach, A. Poustka, and N. Murray.** 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. **170**:827-842.

55. **Gick, O., A. Krämer, W. Keller, and M.L. Birnstiel.** 1986. Generation of histone mRNA 3' ends by endonucleolytic cleavage of the pre-mRNA in a snRNP-dependent in vitro reaction. EMBO J. **5**:1319-1326.

56. **Gorman, C.M., L.F. Moffat, and B.H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044-1051.

57. **Goulian, M., S.H. Goulian, E.E. Codd, and A.Z. Blumenfield.** 1973. Properties of oligodeoxynucleotides that determine priming activity with Escherichia coli deoxyribonucleic acid polymerase I. Biochemistry **12**:2893-2901.

58. **Graham, F.L., and A.J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456-467.

59. **Grandy, D.K., and J.B. Dodgson.** 1987. Structure and organization of the chicken H2B histone gene family. Nucleic Acids Res. **15**:1063-1080.

60. **Grandy, D.K., J.D. Engel, and J.B. Dodgson.** 1982. Complete nucleotide sequence of a chicken H2b histone gene. J. Biol. Chem. **257**:8577-8580.

61. **Graves, R.A., N.B. Pandey, N. Chodchoy, and W.F. Marzluff.** 1987. Translation is required for regulation of histone mRNA degradation. Cell **48**:615-626.

62. **Green, L., R. van Antwerpen, J. Stein, G. Stein, P. Tripputi, B. Emanuel, J. Selden, and C. Croce.** 1984. A major human histone gene cluster on the long arm of chromosome 1. Science **226**:838-840.

63. **Green, M.R.** 1986. Pre-mRNA splicing. Annu. Rev. Genet. **20**:671-708.

64. **Grimes, S., P. Weisz-Carrington, H. Daum III, J. Smith, L. Green, K. Wright, G. Stein, and J. Stein.** 1987. A rat histone H4 gene closely associated with the testis-specific H1t gene. Exp. Cell Res. **173**:534-545.

65. **Gross, K., W. Schaffner, J. Telford, and M. Birnstiel.** 1976. Molecular analysis of the histone gene cluster of Psammechinus miliaris: III. polarity and asymmetry of the histone-coding sequences. Cell **8**:479-484.

66. **Gross-Bellard, M., P. Oudet, and P. Chambon.** 1973. Isolation of high-molecular-weight DNA from mammalian cells. Eur. J. Biochem. **36**:32-38.

67. **Haegeman, G., D. Iserentant, D. Gheysen, and W. Fiers.** 1979. Characterization of the major altered leader sequence of late mRNA induced by SV40 deletion mutant d1-1811. Nucleic Acids Res. **7**:1799-1814.

68. **Hagenbüchle, O., R. Bovey, and R.A. Young.** 1980. Tissue-specific expression of mouse α-amylase genes: nucleotide sequence of isoenzyme mRNAs from pancreas and salivary gland. Cell **21**:179-187.

69. **Haluska, F.G., K. Huebner, M. Isobe, T. Nishimura, C.M. Croce, and P.K. Vogt.** 1988. Localization of the human JUN protooncogene to chromosome region 1p31-32. Proc. Natl. Acad. Sci. USA **85**:2215-2218.

70. **Harvey, R.P., A.J. Robins, and J.R.E. Wells.** 1982. Independently evolving chicken histone H2B genes: Identification of a ubiquitous H2B-specific 5' element. Nucleic Acids Res. **10**:7851-7863.

71. **Harvey, R.P., J.A. Whiting, L.S. Coles, P.A. Krieg, and J.R.E. Wells.** 1983. H2A.F: an extremely variant histone H2A sequence expressed in the chicken embryo. Proc. Natl. Acad. Sci. USA **80**:2819-2823.

72. **Hatch, C.L., and W.B. Bonner.** 1988. Sequence of cDNAs for mammalian H2A.Z, an evolutionarily diverged but highly conserved basal histone H2A isoprotein species. Nucleic Acids Res. **16**:1113-1124.

73. **Hatch, C.L., and W.M. Bonner.** 1990. The human histone H2A.Z gene. J. Biol. Chem. **265**:15211-15218.

74. **Heintz, N., H.L. Sive, and R.G. Roeder.** 1983. Regulation of human histone gene expression: kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during the HeLa cell cycle. Mol. Cell. Biol. **3**:539-550.

75. **Heintz, N., M. Zernik, and R.G. Roeder.** 1981. The structure of the human histone genes: clustered but not tandemly repeated. Cell **24**:661-668.

76. **Helms, S., L. Baumbach, G. Stein, and J. Stein.** 1984. Requirement of protein synthesis for the coupling of histone mRNA levels and DNA replication. FEBS Lett. **168**:65-69.

77. **Hentschel, C.C., and M.L. Birnstiel.** 1981. The organization and expression of histone gene families. Cell **25**:301-313.

78. **Hochhuth, C., and D. Doenecke.** 1990. The expression of the histone H1° gene in the human hepatoma cell line HepG2 is independent of the state of cell proliferation. Differentiation **43**:212-219.

79. **Hoffmann, I., and M.L. Birnstiel.** 1990. Cell cycle-dependent regulation of histone precursor mRNA processing by modulation of U7 snRNA accessibility. Nature **346**:665-668.

80. **Holmes, D.S., and M. Quigley.** 1981. A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**:193-197.

81. **Huebner, K., M. Isobe, J.C. Gasson, D.W. Golde, and C.M. Croce.** 1986. Localization of the gene encoding human erythroid-potentiating activity to chromosome region Xp11.1->Xp11.4. Am. J. Human Genet. **38**:819-826.

82. **Huebner, K., A.P. Palumbo, M. Isobe, C.A. Kozak, S. Monaco, G. Rovera, C.M. Croce, and P.J. Curtis.** 1985. The α-spectrin gene is on chromosome 1 in mouse and man. Proc. Natl. Acad. Sci. USA **82**:3790-3793.

83. **Hwang, I., and C.-B. Chae.** 1989. S-phase-specific transcription regulatory elements are present in a replication-independent testis-specific H2B histone gene. Mol. Cell. Biol. **9**:1005-1013.

84. **Hwang, I., K. Lim, and C.-B. Chae.** 1990. Characterization of the S-phase-specific transcription regulatory elements in a DNA replication-independent testis-specific H2B (TH2B) histone gene. Mol. Cell. Biol. **10**:585-592.

85. **Igo-Kemenes, T., W. Hörz, and H.G. Zachau.** 1982. Chromatin. Annu. Rev. Biochem. **51**:89-121.

86. **Isenberg, I.** 1979. Histones. Annu. Rev. Biochem. **48**:159-191.

87. **Ish-Horowicz, D., and J.F. Burke.** 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9**:2989-2998.

88. **Jackson, V.** 1985. Histone synthesis and deposition in the G1 and S phases of hepatoma tissue culture cells. Biochemistry **24**:6921-6930.

89. **Jarvis, J.W., and W.F. Marzluff.** 1989. The early and late sea urchin histone H4 mRNAs respond differently to inhibitors of DNA synthesis. Dev. Biol. **132**:325-330.

90. **Jung, A., A.E. Sippel, M. Grez, and G. Schütz.** 1980. Exons encode functional and structural units of chicken lysozyme. Proc. Natl. Acad. Sci. USA **77**:5759-5763.

91. **Kedes, L.H.** 1979. Histone genes and histone messengers. Annu. Rev. Biochem. **48**:837-870.

92. **Kemler, I., and W. Schaffner.** 1990. Octamer transcription factors and the cell type-specificity of immunoglobulin gene expression. FASEB J. **4**:1444-1449.

93. **Kim, Y.-J., I. Hwang, L.L. Tres, A.L. Kierszenbaum, and C.-B. Chae.** 1987. Molecular cloning and differential expression of somatic and testes-specific H2B histone genes during rat spermatogenesis. Dev. Biol. **124**:23-34.

94. **Kirsh, A.L., M. Groudine, and P.B. Challoner.** 1989. Polyadenylation and U7 snRNP-mediated cleavage: alternative modes of RNA 3' processing in two avian histone H1 genes. Genes & Development **3**:2172-2179.

95. **Krieg, P.A., and D.A. Melton.** 1984. Formation of the 3' end of histone mRNA by post-translational processing. Nature **308**:203-206.

96. **Krieg, P.A., A.J. Robins, A. Colman, and J.R.E. Wells.** 1982. Chicken histone H5 mRNA: the polyadenylated RNA lacks the conserved histone 3' terminator sequence. Nucleic Acids Res. **10**:6777-6785.

97. **Lawn, R.M., E.F. Fritsch, R.C. Parker, G. Blake, and T. Maniatis.** 1978. The isolation and characterization of linked δ- and β-globin genes from a cloned library of human DNA. Cell **15**:1157-1174.

98. **Leder, P., D. Tiemeier, and L. Enquist.** 1977. EK2 Derivatives of Bacteriophage Lambda useful in the cloning of DNA from higher organisms: The λgtWES System. Science **196**:175-177.

99. **Lehrach, H., D. Diamond, J.M. Wozney, and H. Boedtker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**:4743-4751.

100. **Levenson, R.G., and K.B. Marcu.** 1976. On the existence of polyadenylated histone mRNA in Xenopus laevis oocytes. Cell **9**:311-322.

101. **Levine, B.J., N. Chodchoy, W.F. Marzluff, and A.I. Skoultchi.** 1987. Coupling of replication type histone mRNA levels to DNA synthesis requires the stem-loop sequence at the 3' end of the mRNA. Proc. Natl. Acad. Sci. USA **84**:6189-6193.

102. **Lifton, R.P., M.L. Goldberg, R.W. Karp, and D.S. Hogness.** 1977. The organization of the histone genes in Drosophila melanogaster: functional and evolutionary implications. Cold Spring Harbor Symp. Quant. Biol. **42**:1047-1051.

103. **Little, P.F.R., and S.H. Cross.** 1985. A cosmid vector that facilitates restriction enzyme mapping. Proc. Natl. Acad. Sci. USA **82**:3159-3163.

104. **Liu, T.-J., B.J. Levine, A.I. Skoultchi, and W.F. Marzluff.** 1989. The efficiency of 3'-end formation contributes to the relative levels of different histone mRNAs. Mol. Cell. Biol. **9**:3499-3508.

105. **Lüscher, B., and D. Schümperli.** 1987. RNA 3' processing regulates histone mRNA levels in a mammalian cell cycle mutant. A processing factor becomes limiting in G1-arrested cells. EMBO J. **6**:1721-1726.

106. **Lüscher, B., C. Stauber, R. Schindler, and D. Schümperli.** 1985. Faithful cell-cycle regulation of a recombinant mouse histone H4 gene is controlled by sequences in the 3'-terminal part of the gene. Proc. Natl. Acad. Sci. USA **82**:4389-4393.

107. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159-162.

108. **Maniatis, T., E.F. Fritsch, and J. Sambrook.** 1982. Molecular cloning (a laboratory manual). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

109. **Maniatis, T., R.C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, G.K. Sim, and A. Efstratiadis.** 1978. The isolation of structural genes from libraries of eucaryotic DNA. Cell **15**:687-701.

110. **Mannironi, C., W.M. Bonner, and C.L. Hatch.** 1989. H2A.X. a histone isoprotein with a conserved C-terminal sequence, is encoded by a novel mRNA with both DNA replication type and poly(A) 3' processing signals. Nucleic Acids Res. **17**:9113-9126.

111. **Marashi, F., S. Helms, A. Shiels, S. Silverstein, D.S. Greenspan, G. Stein, and J. Stein.** 1986. Enhancer-facilitated expression of prokaryotic and eukaryotic genes using human histone gene 5' regulatory sequences. Biochem. Cell Biol. **64**:277-289.

112. **Marashi, F., K. Prokopp, J. Stein, and G. Stein.** 1984. Evidence for a human histone gene cluster containing H2B and H2A pseudogenes. Proc. Natl. Acad. Sci. USA **81**:1936-1940.

113. **Marzluff, W.F., and N.B. Pandey.** 1988. Multiple regulatory steps control histone mRNA concentrations. Trends Biochem. Sci. **13**:49-52.

114. **Maxam, A.M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Meth. Enzymol. **65**:499-560.

115. **Messing, J.** 1983. New M13 vectors for cloning. Meth. Enzymol. **101**:20-78.

116. **Molgaard, H.V., M. Perucho, and A. Ruiz-Carrillo.** 1980. Histone H5 messenger RNA is polyadenylated. Nature **283**:502-504.

117. **Morris, T.D., L.A. Weber, E. Hickey, G.S. Stein, and J.L. Stein.** 1991. Changes in the Stability of a human H3 Histone mRNA During the HeLa cell cycle. Mol. Cell. Biol. **11**:544-553.

118. **Mowry, K.L., and J.A. Steitz.** 1987. Both conserved signals on mammalian histone pre-mRNAs associate with small nuclear ribonucleoproteins during 3' end formation in vitro. Mol. Cell. Biol. **7**:1663-1672.

119. **Mowry, K.L., and J.A. Steitz.** 1987. Identification of the human U7 snRNP as one of several factors involved in the 3' end maturation of histone premessenger RNA's. Science **238**:1682-1687.

120. **Mowry, K.L., and J.A. Steitz.** 1988. snRNP mediators of 3' end processing: functional fossils? Trends Biochem. Sci. **13**:447-451.

121. **Murray, N.E., W.J. Brammar, and K. Murray.** 1977. Lambdoid phages that simplify the recovery of in vitro recombinants. Molec. gen. Genet. **150**:53-61.

122. **Nevins, J.R.** 1983. The pathway of eukaryotic mRNA formation. Annu. Rev. Biochem. **52**:441-466.

123. **Norrander, J., T. Kempe, and J. Messing.** 1983. Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene **26**:101-106.

124. **O'Farrell, P.** 1981. Replacement synthesis method of labeling DNA fragments. Focus **3 (3)**:1-3.

125. **Pandey, N.B., and W.F. Marzluff.** 1987. The stem-loop structure at the 3' end of histone mRNAs is necessary and sufficient for regulation of histone mRNA stability. Mol. Cell. Biol. **7**:4557-4559.

126. **Pauli, U., S. Chrysogelos, G. Stein, J. Stein, and H. Nick.** 1987. Protein-DNA interactions in vivo upstream of a cell cycle-regulated human H4 histone gene. Science **236**:1308-1311.

127. **Peltz, S.W., G. Brewer, G. Kobs, and J. Ross.** 1987. Substrate specificity of the exonuclease activity that degrades H4 histone mRNA. J. Biol. Chem. **262**:9382-9388.

128. **Peltz, S.W., and J. Ross.** 1987. Autogenous regulation of histone mRNA decay by histone proteins in a cell-free system. Mol. Cell. Biol. **7**:4345-4356.

129. **Platt, T.** 1986. Transcription termination and the regulation of gene expression. Annu. Rev. Biochem. **55**:339-372.

130. **Plumb, M., J. Stein, and G. Stein.** 1983. Influence of DNA synthesis inhibition on the coordinate expression of core human histone genes during S phase. Nucleic Acids Res. **11**:7927-7945.

131. **Plumb, M., J. Stein, and G. Stein.** 1983. Coordinate regulation of multiple histone mRNAs during the cell cycle in HeLa cells. Nucleic Acids Res. **11**:2391-2410.

132. **Powell, R., J. Neilan, and F. Gannon.** 1986. Plaque Dot Assay. Nucleic Acids Res. **14**:1541-1542.

133. **Prescott, D.M.** 1966. The synthesis of total macronuclear protein, histone, and DNA during the cell cycle in euplotes eurystomus. J. Cell Biol. **31**:1-9.

134. **Prokopp, K.M.** 1984. The nucleotide sequences of a human H2B histone coding region and a portion of its flanking regions. M.Sc. thesis. University of Florida, Gainesville, FL.

135. **Proudfoot, N.J., and G.G. Brownlee.** 1976. 3' non-coding region sequences in eukaryotic messenger RNA. Nature **263**:211-214.

136. **Puchacz, E., J.B. Lian, G.S. Stein, J. Wozney, K. Huebner, and C. Croce.** 1989. Chromosomal localization of the human osteocalcin gene. Endocrinology **124**:2648-2650.

137. **Rackwitz, H., G. Zehetner, A. Frischauf, and H. Lehrach.** 1984. Rapid restriction mapping of DNA cloned in lambda phage vectors. Gene **30**:195-200.

138. **Reed, K.C., and D.A. Mann.** 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. **13**:7207-7221.

139. **Roberts, C., and G.L. Wilson.** 1985. Simplified random priming technique for the radiolabeling of intermediate-sized DNA probes. Focus **7(3)**:16.

140. **Ross, J., and G. Kobs.** 1986. H4 histone messenger RNA decay in cell-free extracts initiates at or near the 3' terminus and proceeds 3' to 5'. J. Mol. Biol. **188**:579-593.

141. **Ross, J., G. Kobs, G. Brewer, and S.W. Peltz.** 1987. Properties of the exonuclease activity that degrades H4 histone mRNA. J. Biol. Chem. **262**:9374-9381.

142. **Ross, J., S.W. Peltz, G. Kobs, and G. Brewer.** 1986. Histone mRNA degradation in vivo: the first detectable step occurs at or near the 3' terminus. Mol. Cell. Biol. **6**:4362-4371.

143. **Ruberti, I., P. Fragapane, P. Pierandrei-Amaldi, E. Beccari, F. Amaldi, and I. Bozzoni.** 1982. Characterization of histone genes isolated from Xenopus laevis and Xenopus tropicalis genomic libraries. Nucleic Acids Res. **10**:7543-7557.

144. **Ruderman, J.V., and M.L. Pardue.** 1978. A portion of all major classes of histone messenger RNA in amphibian oocytes is polyadenylated. J. Biol. Chem. **253**:2018-2025.

145. **Salser, W.** 1977. Globin mRNA sequences: analysis of base pairing and evolutionary implications. Cold Spring Harbor Symp. Quant. Biol. **42**:985-1002.

146. **Sanger, F., S. Nicklen, and A.R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463-5467.

147. **Schaufele, F., G.M. Gilmartin, W. Bannwarth, and M.L. Birnstiel.** 1986. Compensatory mutations suggest that base-pairing with a small nuclear RNA is required to form the 3' end of H3 messenger RNA. Nature **323**:777-781.

148. **Scherrer, K., and J. Darnell.** 1962. Sedimentation characteristics of rapidly labelled RNA from HeLa cells. Biochem. Biophys. Res. Commun. **7**:486-490.

149. **Schweizer, H.P.** 1988. An improved procedure for the rapid purification of bacteriophage lambda DNA from small phage lysates. DNA and Protein Engineering Techniques **1**:35-36.

150. **Schümperli, D.** 1986. Cell cycle regulation of histone gene expression. Cell **45**:471-472.

151. **Schümperli, D.** 1988. Multilevel regulation of replication-dependent histone genes. Trends Genet. **4**:187-191.

152. **Shalhoub, V., L.C. Gerstenfeld, D. Collart, J.B. Lian, and G.S. Stein.** 1989. Downregulation of cell growth and cell cycle regulated genes during chick osteoblast differentiation with the reciprocal expression of histone gene variants. Biochemistry **28**:5318-5322.

153. **Shiels, A., F. Marashi, G. Stein, and J. Stein.** 1985. Enhancer-facilitated expression of a human H4 gene. Biochem. Biophys. Res. Commun. **127**:239-246.

154. **Sierra, F., A. Lichtler, F. Marashi, R. Rickles, T. van Dyke, S. Clark, J. Wells, G. Stein, and J. Stein.** 1982. Organization of human histone genes. Proc. Natl. Acad. Sci. USA **79**:1795-1799.

155. **Sittman, D.B., R.A. Graves, and W.F. Marzluff.** 1983. Histone mRNA concentrations are regulated at the level of transcription and mRNA degradation. Proc. Natl. Acad. Sci. USA **80**:1849-1853.

156. **Soldati, D., and D. Schümperli.** 1988. Structural and functional characterization of mouse U7 small nuclear RNA active in 3' processing of histone pre-mRNA. Mol. Cell. Biol. **8**:1518-1524.

157. **Southern, E.M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.

158. **Southern, P.J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327-341.

159. **Stauber, C., B. Lüscher, R. Eckner, E. Lötscher, and D. Schümperli.** 1986. A signal regulating mouse histone H4 mRNA levels in a mammalian cell cycle mutant and sequences controlling RNA 3' processing are both contained within the same 80-bp fragment. EMBO J. **5**:3297-3303.

160. **Stauber, C., and D. Schümperli.** 1988. 3' processing of pre-mRNA plays a major role in proliferation-dependent regulation of histone gene expression. Nucleic Acids Res. **16**:9399-9414.

161. **Stein, G., J. Lian, J. Stein, R. Briggs, V. Shalhoub, K. Wright, U. Pauli, and A. van Wijnen.** 1989. Altered binding of human histone gene transcription factors during the shutdown of proliferation and onset of differentiation in HL-60 cells. Proc. Natl. Acad. Sci. USA **86**:1865-1869.

162. **Stein, G.S., and T.W. Borun.** 1972. The synthesis of acidic chromosomal proteins during the cell cycle of HeLa S-3 cells. J. Cell Biol. **52**:292-307.

163. **Stein, G.S., and J.L. Stein.** 1984. The relationship between human histone gene expression and DNA replication. BioEssays **1**:202-205.

164. **Stein, G.S., J.L. Stein, and W.F. Marzluff (eds.).** 1984. Histone genes. John Wiley & Sons, New York.

165. **Stent, G.S., and R. Calendar.** 1971. Molecular genetics: An introductory narrative. W.H. Freeman and Company, San Francisco, CA.

166. **Strub, K., and M.L. Birnstiel.** 1986. Genetic complementation in the Xenopus oocyte: Co-expression of sea urchin histone and U7 RNAs restores 3' processing of H3 mRNA in the oocyte. EMBO J. **5**:1675-1682.

167. **Strub, K., G. Galli, M. Busslinger, and M.L. Birnstiel.** 1984. The cDNA sequences of the sea urchin U7 small nuclear RNA suggest specific contacts between histone mRNA precursor and U7 RNA during RNA processing. EMBO J. **3**:2801-2807.

168. **Tabor, S., H.E. Huber, and C.C. Richardson.** 1987. Escherichia coli Thioredoxin confers processivity on the DNA polymerase activity of the gene 5 protein of bacteriophage T7. J. Biol. Chem. **262**:16212-16223.

169. **Taylor, J.M., R. Illmensee, and J. Summers.** 1976. Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochim. Biophys. Acta **442**:324-330.

170. **Thomas, P.S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201-5205.

171. **Tripputi, P., B.S. Emanuel, C.M. Croce, L.G. Green, G.S. Stein, and J.L. Stein.** 1986. Human histone genes map to multiple chromosomes. Proc. Natl. Acad. Sci. USA **83**:3185-3188.

172. **Tsai, Y.H., and L.S. Hnilica.** 1975. Tissue-specific histones in the erythrocytes of chicken and turtle. Exp. Cell Res. **91**:107-112.

173. **Tsuchiya, S., Y. Kobayashi, Y. Goto, H. Okumura, S. Nakae, T. Konno, and K. Tada.** 1980. Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. Int. J. Cancer **26**:171-176.

174. **Tullis, R.H., and H. Rubin.** 1980. Calcium protects DNase I from proteinase K: A new method for the removal of contaminating RNase from DNase I. Anal. Biochem. **107**:260-264.

175. **Urban, M.K., S.G. Franklin, and A. Zweidler.** 1979. Isolation and characterization of the histone variants in chicken erythrocytes. Biochemistry **18**:3952-3960.

176. **van Wijnen, A.J., R.F. Massung, J.L. Stein, and G.S. Stein.** 1988. Human H1 histone gene promoter CCAAT box binding protein HiNF-B is a mosaic factor. Biochemistry **27**:6534-6541.

177. **van Wijnen, A.J., K.L. Wright, R.F. Massung, M. Gerretsen, J.L. Stein, and G.S. Stein.** 1988. Two target sites for protein binding in the promoter region of a cell cycle regulated human H1 histone gene. Nucleic Acids Res. **16**:571-592.

178. **Vasserot, A.P., I. Hoffmann, K. Tabiti, and M.L. Birnstiel.** 1990. 3' Processing of histone RNA precursors. Mol. Bio. Reports. **14**:211-212.

179. **Vasserot, A.P., F.J. Schaufele, and M.L. Birnstiel.** 1989. Conserved terminal hairpin sequences of histone mRNA precursors are not involved in duplex formation with the U7 RNA but act as a target site for a distinct processing factor. Proc. Natl. Acad. Sci. USA **86**:4345-4349.

180. **Wahl, G.M., M. Stern, and G.R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. USA **76**:3683-3687.

181. **Waxman, D.J., and J.L. Strominger.** 1983. Penicillin-binding proteins and the mechanism of action of β-lactam antibiotics. Annu. Rev. Biochem. **52**:825-869.

182. **Wells, D., D. Hoffman, and L. Kedes.** 1987. Unusual structure, evolutionary conservation of non-coding sequences and numerous pseudogenes characterize the human H3.3 histone multigene family. Nucleic Acids Res. **15**:2871-2889.

183. **Wells, D., and L. Kedes.** 1985. Structure of a human histone cDNA: evidence that basally expressed histone genes have intervening sequences and encode polyadenylylated mRNAs. Proc. Natl. Acad. Sci. USA **82**:2834-2838.

184. **Wells, D.E.** 1986. Compilation analysis of histones and histone genes. Nucleic Acids Res. **14**:r119-r149.

185. **Woudt, L.P., A. Pastink, A.E. Kempers-Veenstra, A.E.M Jansen, W.H. Mager, and R.J. Planta.** 1983. The genes coding for histone H3 and H4 in Neurospora crassa are unique and contain intervening sequences. Nucleic Acids Res. **11**:5347-5360.

186. **Wu, M., D.S. Holmes, and N. Davidson.** 1976. The relative positions of sea urchin histone genes on the chimeric plasmid pSp2 and pSp17 as studied by electron microscopy. Cell **9**:163-169.

187. **Wu, R.S., and W.M. Bonner.** 1981. Separation of basal histone synthesis from S-phase histone synthesis in dividing cells. Cell **27**:321-330.

188. **Wu, R.S., S. Tsai, and W.M. Bonner.** 1982. Patterns of histone variant synthesis can distinguish G0 from G1 cells. Cell **31**:367-374.

189. **Yamamoto, K.R., B.M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber.** 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology **40**:734-744.

190. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103-119.

191. **Young, R.A., and R.W. Davis.** 1983. Yeast RNA polymerase II genes: Isolation with antibody probes. Science **222**:778-782.

192. **Zambetti, G., W. Schmidt, G. Stein, and J. Stein.** 1985. Subcellular localization of histone messenger RNAs on cytoskeleton-associated free polysomes in HeLa S_3 cells. J. Cell. Physiol. **125**:345-353.

193. **Zambetti, G., J. Stein, and G. Stein.** 1987. Targeting of a chimeric human histone fusion mRNA to membrane-bound polysomes in HeLa cells. Proc. Natl. Acad. Sci. USA **84**:2683-2687.

194. **Zhong, R., R.G. Roeder, and N. Heintz.** 1983. The primary structure and expression of four cloned human histone genes. Nucleic Acids Res. **11**:7409-7425.

195. **Zweidler, A.** 1980. Nonallelic histone variants in development and differentiation. Dev. Biochem. **15**:47-56.

196. **Zwollo, P., G.S. Stein, and J.L. Stein.** 1984. Variations in the organization of human genomic DNA segments containing H1 histone genes. Biochem. Biophys. Res. Commun. **124**:988-993.

BIOGRAPHICAL SKETCH

David George Collart was born in Weirton, West Virginia, on May 22, 1961. He attended Blessed Sacrament School in Wintersville, Ohio, from 1967 to 1968 and St. Peter's school in Huron, Ohio, from 1968 to 1975. He attended Edison High School (Milan, Ohio) and graduated in 1979. He then entered Firelands College of Bowling Green State University (Huron, Ohio) in the fall of 1979, transferred to the main campus at Bowling Green, Ohio, in the fall of 1980 and graduated with a Bachelor of Science degree in chemistry in the summer of 1983. He began research in the laboratory of Drs. Gary and Janet Stein and, in the fall of 1983, he entered the graduate program in the Department of Biochemistry and Molecular Biology at the University of Florida. On August 1, 1987, he married Kathleen Leah Dudeck in Gainesville, Florida, and on October 5, 1990, God blessed them with the birth of a son, Timothy Gerard Collart. After graduation, he will pursue research as a postdoctoral fellow with Dr. Gray Crouse in the Department of Biology at Emory University in Atlanta, Georgia.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Gary S. Stein, Chair
Professor of Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Janet L. Stein, Co-chair
Professor of Immunology and
Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Philip J. Lapis
Professor of Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Harry S. Nick
Associate Professor of
Biochemistry and Molecular
Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Harry Ostrer
Associate Professor of
Biochemistry and Molecular
Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Carl Feldherr
Professor of Anatomy

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Lee A. Weber
Professor of Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Eileen Hickey
Professor of Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 1991

Dean, College of Medicine

Dean, Graduate School